

POSITIVE AND NEGATIVE DYNAMICS OF PLANT-PLANT
INTERACTIONS ALONG ENVIRONMENTAL GRADIENTS:
EFFECTS AT INDIVIDUAL AND COMMUNITY LEVEL

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ZUSAMMENFASSUNG

Ergebnisse großer Grünland-Biodiversitätsexperimente legen einen positiven Zusammenhang zwischen Artenvielfalt und Ökosystemfunktionen (z.B. Produktivität, Ressourcennutzung, Stabilität der Gemeinschaften) nahe. Der durch Klima- und Landnutzungswandel andauernde Artenverlust bedroht die Bereitstellung dieser Funktionen. Und obschon die Zusammenhänge zwischen Artenreichtum und Ökosystemfunktionen, die man auf Gemeinschaftsebene beobachten kann, abhängig von Interaktionen zwischen Individuen und Arten sind, sind Informationen über diese Mechanismen rar.

Die vorliegende Dissertation stellt fünf Projekte vor, die sich mit Mechanismen der Pflanze-Pflanze-Interaktion am Beispiel von Grünlandarten der gemäßigten Breiten befassen, speziell mit der Leguminose-Nachbar-Interaktion. Auf der Ebene von Individuen und Populationen wurden Veränderungen der Interaktionen untersucht: entlang von biotischen Gradienten (Vielfalt, Zusammensetzung und Identität der Arten einer Gemeinschaft), entlang von abiotischen Gradienten (Extremwetterereignisse, Stickstoffverfügbarkeit) und in Einheiten verschiedener räumlicher Abmessungen. Drei Leitfragestellungen motivierten die Projekte: Ist das Testen ökologische Theorien, die von großräumigen Beobachtungen abgeleitet wurden, auch in kleinräumigen Einheiten möglich? Gibt es eine Grenze, an der positive Leguminosen-Effekte (*N-facilitation*) auf den Stickstoffhaushalt der Nachbarn (*receiver*), in Konkurrenz um andere Ressourcen umschlagen? Wie wirken sich Anzahl und Identitäten der Arten einer Gemeinschaft auf artspezifische Interaktionen aus? Zur Beantwortung dieser Fragen habe ich traditionelle, invasive und nicht-invasive Methoden benutzt.

Die in der Dissertation vorgestellten Studien belegen die Möglichkeit, „Feld-Effekte“ auch in kleineren räumlichen Einheiten zu untersuchen, da zahlreiche Übereinstimmungen zwischen Untersuchungen im Feld und in Mikrokosmen auftraten. So konnten wir die Abnahme der $\delta^{15}\text{N}$ Werte mit abnehmender N-Versorgung und zunehmender Artenzahl, die unserem Wissen nach bisher ausschließlich in Feldversuchen nachgewiesen wurde, in Mikrokosmen feststellen. Ebenso konnten wir zum Feld vergleichbare positive Effekte zunehmender Artenzahlen und vorhandener N_2 -Fixierer auf Interaktionen zwischen Pflanzen in ungestörten Gemeinschaften nachweisen. *N-facilitation* finden über N-Transfer von N_2 -Fixierern zu *receivers* und über Ressourcenumverteilung des Boden-N (*N-sparing*) statt. Kurzfristig bot *N-sparing* den größeren Vorteil für *receiver*; wir haben aber ebenso kurzfristigen N-Transfer zwischen Arten verschiedener Identitäten nachgewiesen. Die Artidentität spielt für die Nutzung des durch Leguminosen bereitgestellten Extra-N aus *N-facilitation* eine wesentliche

Rolle: Gräser nutzten Extra-N aus *N-sparing* und N-Transfer effektiver als krautige Arten, sowohl im Feld als auch in Mikrokosmen. Darüber hinaus haben wir neue Muster des Einflusses der Artenzahl auf artspezifische N-Dynamiken in gestörten Gemeinschaften entdeckt. In Mikrokosmen mit einer Gras-, Kraut- und Leguminosen-Art stieg in Folge simulierter Beweidung der N-Transfer zwischen Individuen in Monokulturen unabhängig von der Artidentität an, nahm in Mischungen hingegen ab. Eine völlig andere applizierte Störung (Trockenstress statt Beweidung) resultierte in einem ähnlichen Muster bezüglich der N-Parameter eines Grases in unterschiedlichen Artgemeinschaften im EVENT-Experiment. Die Abnahme von *N-facilitation* nach Störungen könnte eine Erklärung für die verminderte Leistung des Grases in artenreicheren Gemeinschaften sein, auch wenn mit nicht-invasiven (und invasiven) Methoden auf Ebene der Gemeinschaft keine gravierenden negativen Effekte feststellbar waren. Diese Konstanz auf einer höheren Organisationsebene legt nahe, dass die verwendeten nicht-invasive Methoden neue Möglichkeit eröffnen, ökologische Theorien auf der Ebene von Artengemeinschafts (z.B. *insurance hypothesis*, „Versicherungshypothese“) in unterschiedlichen räumlichen Einheiten zu untersuchen.

Die Ergebnisse lassen den Rückschluss zu, dass Versuche unter kontrollierten Umweltbedingungen in kleineren räumlichen Einheiten ein geeignetes Mittel sind, um Effekte und Interaktionen von Faktoren (Vielfalt, Zusammensetzung, Identität der Arten einer Gemeinschaft) detailliert zu untersuchen. Insbesondere die vergleichbare N-Dynamik in Feld- und Mikrokosmosversuchen scheint das Testen mancher ökologischer Theorien, z.B. der *stress gradient hypothesis*, in kleinräumigen Einheiten zu ermöglichen. Darüber hinaus wird im Rahmen der Dissertationsschrift die Nutzbarkeit verschiedener invasiver und nicht-invasiver Methoden zur Untersuchung von Pflanze-Pflanze-Interaktionen dargestellt. Diese Ergebnisse bilden das Fundament für weiterführende Projekte, die dazu beitragen sollen, Zusammenhänge zwischen Biodiversitätseffekten und Ökosystemfunktionen zu erhellen und damit z.B. eine kosteneffektive Renaturierung degradierter Standorte zu erleichtern.

SUMMARY

Biodiversity and the functioning of communities, habitats or even ecosystems are closely connected as worldwide large-scale biodiversity grassland experiments reveal. Current climate and land use changes are often related to a loss of plant species diversity from natural grassland habitats and as a consequence, the delivery of “ecosystem functions” (e.g. productivity, stability against disturbance or total nutrient use) is endangered. But information about the underlying mechanisms, which drive relationships between biodiversity and these functions, is still missing, although all processes observable at community scale depend on processes between species or individuals *within* communities. The study of plant-plant interactions, with a special focus on legume-neighbour interactions, within grassland habitats of the temperate region is the main focus of this thesis.

I investigated legume-neighbour interactions on individual and population level (i) along biotic gradients of community composition (species richness and species identity), (ii) along abiotic gradients (extreme weather events, nitrogen availability) and (iii) at different spatial scales (from the climate chamber to the field). Three main research questions linked individual projects: Is it possible to test ecological theories, which are derived from large-scale observations, on a much smaller scale? Is it possible to identify a threshold, where positive effects of nitrogen-fixing legume species (N-facilitation) shift to competition for above- or belowground resources? How does community diversity modulate species-specific plant-plant interactions? To answer these questions I used different invasive and non-invasive methods like the analyses of the isotopic composition of N or chlorophyll *a* fluorescence in different species as well as traditional ecological census techniques.

Results from the five studies presented within this thesis (manuscripts 1-4 and Supplementary Material) provide strong evidence that it is indeed possible to simulate field-effects at a much smaller scale because multiple similarities occurred between field studies and studies at smaller scales. In micro- and mesocosm studies, we were able to confirm the decrease in $\delta^{15}\text{N}$ natural abundance values with decreasing N-availability in the substrate and with increasing species richness, as it has been reported, to our knowledge, exclusively from field studies. We found positive effects of increasing species richness on plant-plant interactions and positive effects of legume presence (N-facilitation either by N-sparing or by N-transfer) on N-availability for neighbouring receiver species in undisturbed communities, which are comparable to field observations. In the short-term, receivers profited mostly from N-sparing but in addition a bidirectional N-transfer between functionally different individuals occurred. We were also

able to simulate differences in the use of extra N from N-facilitation in relation to the identity of receiver species, which are known from field studies: grass species in microcosms showed a better use of extra N from N-facilitation (both N-sparing and N-transfer) compared to non-fixing forb species. Furthermore, we found a totally novel pattern of the modulating effect of species diversity on species-specific N-dynamics after disturbance: whereas N-transfer increased in monocultures, it decreased in mixtures after simulated grazing in microcosm communities. Although treated with a totally different disturbance (extreme drought event), N-parameters of a common grass species in different diversity levels in the EVENT-Experiment indicate a similar pattern. This is a possible explanation for a non-invasively detected performance reduction (via measurements of chlorophyll *a* fluorescence) of this species, although at community level no negative effects of increased species richness were observed. Relative constant community fluorescence signals provide first evidence, that it is possible to use fluorescence measurements as a non-invasive method to test the insurance hypothesis.

These findings imply that studies on smaller scales under controlled environmental conditions are very useful to test effects of species richness and identity as well as ecological theories. Patterns of N-dynamics in microcosms resemble those observed in field experiments and thus, some theories (e.g. the stress gradient hypothesis) are indeed testable on a much smaller scale. I provide novel insights on changes in plant-plant interactions within different abiotic and biotic environments and to what extent different invasive and non-invasive methods are useful to elucidate interaction processes. Further research on plant-plant interactions is needed, e.g. with regard to the cost-effective restoration of degraded grassland habitats.

INTRODUCTION

BACKGROUND OF THE THESIS

BIODIVERSITY EFFECTS ON ECOSYSTEM FUNCTIONING

Large-scale biodiversity experiments like the Jena Experiment in Germany (Roscher et al. 2004; Roscher et al. 2005), the Paneuropean BIODEPTH experiments (Hector et al. 1999; Hector et al. 2007) or the Cedar Creek Experiment in the USA (Tilman 1987; Zavaleta et al. 2010) contribute considerably to our understanding of grassland ecosystems. The experiments reveal positive relationships between plant species/functional diversity and ecosystem functions such as (i) productivity, (ii) stability and resistance against (alien) species invasion or environmental disturbances, (iii) recovery after disturbances and (iv) total resource use. And although a diversity of four to ten species is often enough to maintain a single function, e.g. productivity *or* stability (Schwartz et al. 2000; Guo et al. 2006; van Ruijven and Berendse 2009), much higher diversity may be required to sustain multifunctionality, e.g. productivity *and* stability (Hector and Bagchi 2007; Zavaleta et al. 2010). Additionally, recent investigations highlight the importance of genetic diversity for ecosystem functioning (Hughes et al. 2008; Agashe 2009; Vellend et al. 2010) and the importance of plant diversity for subsequent trophic levels like soil organisms, pollinators, herbivores or predators of herbivores (De Deyn and van der Putten 2005; Duffy et al. 2007; Evans 2008) and also for human well-being (Diaz et al. 2006; Fuller et al. 2007). Positive biodiversity-ecosystem functioning relationships are summarized in influential ecological theories like the insurance hypothesis (McNaughton 1977; Naeem et al. 1994; Yachi and Loreau 1999) or the niche complementary theory (Berendse 1979; Tilman 1997; Loreau and Hector 2001).

The insurance hypothesis predicts that functioning of an ecosystem under disturbance will be better maintained in more diverse communities, with a higher potential for trait redundancy, than in less diverse communities. For example, if species A and B get extinct from a species-rich community (because they suffer from climate change induced drought stress), species C, D, E and F can buffer ecosystem productivity against negative effects whereas communities consisting only of species A and B will totally collapse. The niche complementarity theory predicts that more diverse communities, consisting of species with different spatial and temporal acquisition strategies, will exploit available resources (e.g. belowground water and nutrients, aboveground light) more complete and more effective than less diverse communities. An important issue for the niche complementarity theory is the interplay between plants; the equilibrium between positive interactions (facilitation) and negative

interactions (competition). Facilitation, *sensu* Connell & Slatyer (1977), is the ability of one species to modify the environment beneficially for another species, whereby one species has a positive effect on neighbouring or subsequent species; although there is an ongoing discussion about the exact definition of facilitation (Brooker and Callaway 2009). Legume-neighbour interactions provide an comprehensive example for the equilibrium of interactions: under nitrogen (N) limited conditions, legume species have a positive effect on neighbours by providing extra nitrogen from biological nitrogen fixation (BNF) (facilitation for N-nutrition) but on N-saturated soils they can have negative effects on neighbours due to their fast growth and high biomass production (competition for light). Critical voices often state that positive relationships observed in biodiversity experiments are mainly due to the species pool chosen, which often includes an artificially high presence of key species (highly productive species or species otherwise responsible for the ecosystem service under observation) and that *those* species are the driver of positive diversity effects and not the diversity *per se*, a theory summarized as “sampling effect” (or selection effect) (Aarssen 1997; Huston 1997). Recent research indicate, that the importance of the sampling effect for the delivery of a certain ecosystem function (productivity) might be high in young communities, but that, in the long-term, the effect size of complementarity increases whereas the effect size of sampling effects decreases (Marquard et al. 2009a).

Although lots of energy has been spent during the last 20 years to elucidate details of biodiversity-ecosystem functioning relationships, we are still lacking knowledge about the underlying mechanisms, which cause these positive effects. Most studies on facilitation, which provide a more detailed insight into interaction processes, were inspired by economic-agricultural questions and thus were performed with low species diversity; investigations with two species in (more or less) eutrophic environments are most common. During my PhD, I investigated plant-plant interactions, with a special focus on legume-neighbour interactions, and how they affect productivity and resource use efficiency (light: **manuscript 1, manuscript 2**, nitrogen: **manuscript 3, manuscript 4**) along biotic gradients (diversity/species composition: **manuscript 1, manuscript 2, manuscript 4, supplementary material**) and abiotic gradients (resource availability: **manuscript 3, supplementary material**; disturbance: **manuscript 1, manuscript 2, manuscript 4**). My aim was to provide a link between previous results from large-scale field experiments and more mechanistic, physiologically detailed studies under controlled conditions and to test the applicability of ecological theories at different scales.

LEGUME EFFECTS ON N-DYNAMICS AND PERFORMANCE OF NEIGHBOURS

In 1888, Hellriegel and Wilfarth were the first authors who described the symbiosis between N₂-fixing bacteria and legume species, which is responsible for biological nitrogen fixation (BNF) of atmospheric N₂ in legumes (Marschner 2002). Positive legume effects play a key role for agricultural yield production since ancient times and are still an important topic in modern sustainable agriculture. Much effort was spent on the study of legume-neighbour interactions, mainly in agricultural research, to understand and optimize BNF and N-facilitation. Effects on productivity and N-availability have been reported for agricultural pasture (McNeill and Wood 1990b; Elgersma et al. 2000) and crop cultivation (Fujita et al. 1992; Varvel and Wilhelm 2003; Li et al. 2007) including context-dependent information about the relationship between the amount of N-facilitation and climatic or edaphic conditions (Giller and Cadisch 1995, see **manuscript 3**). The ability to perform BNF classifies legume species as ecosystem engineers *sensu* Jones et al. (1994) because they alter their abiotic environment by shifting N from the atmosphere to the soil; an effect, which reaches far beyond agricultural questions.

In every ecosystem, the presence of legume species affects the total amount of niches positively and thus often facilitates increasing population, community or even ecosystem processes. Legume species (acting as N-donors) can affect N-availability in the soil for neighbouring or subsequent species (N-receivers) directly via the exudation of N-rich compounds (Ayers and Thornton 1968; Paynel and Cliquet 2003), decomposition of their own (mostly N-rich) tissue and enhanced total decomposition (Russell and Fillery 1996; Fillery 2001; Scherer-Lorenzen 2008). They also can increase N-availability for neighbours indirectly by *not* using soil resources, an effect known as N-sparing (e.g. McNeill and Wood 1990a). Furthermore, legume species often interact with other trophic levels like soil microorganisms (Habekost et al. 2008; Kreyling et al. 2008b), mycorrhizal fungi (Jackson et al. 2008) or earthworms (Eisenhauer et al. 2009) enhancing their own effects on neighbours and ecosystems even further.

In biodiversity experiments, legume species often count as key species because of their ability to sustain their own N-demand by BNF and their often superior productivity; but positive biodiversity-productivity relationships have also been observed without legume species (summarized in van Ruijven and Berendse 2009). Productivity had long been the only response parameter to measure positive legume effects but since the 1970's the establishment of more elaborated analysis methods provides tools to track the flow of nitrogen through a

system or different trophic levels, e.g. the analysis of the isotopic composition of nitrogen in a sample (see Shearer and Kohl 1986 and references within). Biodiversity experiments in mesic grasslands provide evidence that positive legume effects (N-facilitation), reflected in the N-status and often in the isotopic composition of non-fixing receiver species, contribute to positive biodiversity effects on community productivity and nutrient cycling (Carlsson et al. 2009; Mulder et al. 2002; Spehn et al. 2002; Temperton et al. 2007). Temperton et al. (2007) found strong facilitative interactions between three different mesic grassland species (receivers) and neighbouring legume species (donors) along a gradient of plant species diversity in a field experiment. They found that donor presence (but interestingly not abundance) affects N-concentration and N-content as well as the isotopic composition of N in receivers, but also that an increasing number of surrounding species decreased N-concentration and the relative amount of ^{15}N . Important information is still missing on the mechanisms of legume-neighbour interactions and how they change along biotic and abiotic gradients. I contribute information to the field of legume-neighbour interactions under different conditions of N-availability in a restoration project (**manuscript 3**) and a microcosm study (**supplementary material**) and how changes in species composition and management regime (simulation of grazing) under stable abiotic conditions affect N-facilitation for functionally different receivers (**manuscript 4**).

PLANT-PLANT INTERACTIONS ALONG ENVIRONMENTAL GRADIENTS

Species' performance and inter-specific interactions depend strongly on the broader environmental context in which they are measured (Michalet et al. 2006; Cardinale et al. 2009; Ma et al. 2010). *Within* a defined abiotic environment, plant species can interact via competition or facilitation (Pugnaire and Luque 2001; Brooker et al. 2008). Since publication of Darwin's "The Origin of Species" in 1859, competition between species had been used as the main factor explaining community structure – although facilitation had been identified as potentially important in succession theory in the early 20th century (reviewed in Connell and Slatyer 1977). In 1994, Bertness and Callaway formulated the stress gradient hypothesis (SGH) (Bertness and Callaway 1994) which, for the first time, includes facilitation as an aspect affecting community structure along environmental gradients. Today this is a widely accepted concept (Bruno et al. 2003; Michalet et al. 2006; Brooker et al. 2008; Bulleri 2009) although competition is still widely considered to be the main driver of community structure. The SGH predicts equilibrium between positive and negative interactions along

environmental gradients: whereas negative interactions prevail at the mesic/favourable end of an environmental gradient, positive interactions gain in influence with increasing environmental severity. To revisit an earlier example: under N-saturated conditions, vigorously growing legume species can have significant negative effects on neighbouring species due to space and light competition but under N-limited conditions, the same neighbouring species may profit from a legume species due to N-facilitation. During the last decades, the SGH has experienced support (Pugnaire and Luque 2001; Arredondo-Nunez et al. 2009) as well as criticism (Maestre et al. 2006). Recently, a consensus has been achieved: the SGH generally holds true if pair-wise species-specific investigations are evaluated but might not allow for general predictions of the frequency and kind of interactions (Maestre et al. 2009; le Roux and McGeoch 2010).

Most studies which tested the SGH use facilitation in terms of nurse plant effects (a resident plant enables seedlings of a different species to establish and flourish underneath it by providing shelter or increased resource availability) in climatically extreme arid or alpine ecosystems. Only few studies investigate N-facilitation by legume species and changes in legume-neighbour interactions along abiotic or biotic gradients in communities of varying species richness in benign ecosystems, although these systems dominate the temperate regions of Europe. Fertiliser studies in European mesic pastures often report productivity preservation if legume species are present despite severely reduced N-addition or N-removal by harvest without subsequent fertilisation (e.g. Ledgard et al. 2001; Marquard et al. 2009a; Weigelt et al. 2009). A theoretical link between SGH and fertiliser studies suggests that N-facilitation should increase with increasing N-limitation even in less extreme habitats. These changes in legume-neighbour interactions should be detectable via the analysis of the isotopic composition of nitrogen in non-legume receiver species as described in detail in **manuscript 3**. Only few studies strike this path and investigate N-facilitation under (semi-)natural conditions within the temperate regions. Beyschlag et al. (2009) investigated N-facilitation of legume species on receivers in German dry acidic grassland communities and Temperton et al. (2007) investigated N-facilitation along a biotic gradient of species richness in mesic grassland communities within the Jena Experiment – but both studies lack an abiotic gradient in e.g. N-availability in the substrate. I aim to reduce the lack of information on changes in plant-plant interactions with changing environmental conditions with the studies presented within this thesis; effects of extreme weather events (**manuscript 1**, **manuscript 2**) and effects of the N-availability, ranging from severely N-limited to mesic (**manuscript 3**, **supplementary material**) were investigated.

Furthermore, only few studies explicitly tested the interplay between the SGH and the insurance hypothesis. In combination, these hypotheses predict higher stability of more diverse communities in the face of disturbance due to functional redundancy of species with additional effects of higher facilitation under environmental stress. The combination of both ecological theories raises the questions how an increase in environmental stress (e.g. due to ongoing climate change) will affect legume-neighbour interactions within the temperate regions and how community composition and species diversity will modulate these interaction processes on individual and population level. Investigations of interactive biodiversity and legume effects make considerably requirements on the experimental design and only few set ups meet the demands. The working groups around R. Ceulemans and H. J. de Boeck in Belgium (e.g. De Boeck et al. 2006; Lemmens et al. 2006; De Boeck et al. 2007) and around A. Jentsch and C. Beierkuhnlein at the EVENT-Experiments in Germany (e.g. Jentsch et al. 2007; Kreyling et al. 2008b; Kreyling et al. 2008c) investigate, amongst others, the effect of legume species on ecosystem processes. In **manuscript 1** and **manuscript 2**, I report about studies which investigated effects of community composition and legume presence on the performance of a common grass species under differently severe environmental conditions.

Another aspect of disturbance *per se* is the land use regime (grazing, mowing or habitat restoration) applied to semi-natural grasslands. Again, very little information is available about changes in legume-neighbour interactions with changes in the management. Performance of receiver species within differently treated areas in a large-scale restoration project should provide support for the SGH in terms of N-facilitation (**manuscript 3**). Effects of diversity level and species composition on appearance and changes in small-scale donor-receiver interactions are highlighted in a study under controlled environmental conditions disturbed by simulated grazing (**manuscript 4**).

PLANT-PLANT INTERACTIONS ALONG SPATIAL GRADIENTS

“Scale is fundamental in ecology because it determines how we perceive patterns and processes, and therefore affects our ability to explain and predict” (in Sandel and Smith 2009 from Wiens 1989). Since the late 1980’s, scale-dependency of processes received increasing attention in ecological studies, especially with the aim to scale up from smaller experimental units to larger, ecosystem relevant units (reviewed by Sandel and Smith 2009). Problems in comparing small-scale studies with studies from a larger scale had been identified soon (first-

time reviewed by Wiens 1989). Balvanera et al. (2006) state that effects of plant diversity on ecosystem processes strongly depend on the observation level and on the degree of manipulation in an experimental set-up. On ecosystem level manipulations often have only minor effects but biodiversity itself has strong positive effects, whereas on population, species or even individual level manipulations often have strong effects and biodiversity can have negative effects. Using the example of productivity, most studies on larger spatial levels (e.g. communities of a certain habitat) found a prevailing positive effect of biodiversity and just a subordinate negative effect of a manipulation (e.g. a drought treatment) on productivity (Tilman and Downing 1994; Grime et al. 2008). Whereas on a smaller spatial level (e.g. a certain species within a community), the surrounding plant diversity might affect species-specific biomass production negatively (van Ruijven and Berendse 2003; Roscher et al. 2007; Marquard et al. 2009b) and the effect of a manipulation depends strongly on plant-plant interactions and the competitive strength of the species under investigation (see **manuscript 1**, **manuscript 2**). Thus, investigations at two different spatial scales may result in contrasting findings and a scientist should always be aware of the scale-dependency of response parameters.

But nevertheless, it *is* possible to simulate habitat-related processes in experimental plots of severely reduced size in the field (e.g. for mesic grasslands see Roscher et al. 2005; Hector et al. 2007; Kreyling et al. 2008c; Marquard et al. 2009a) or even in greenhouse experiments (Lanta and Leps 2006), although it is widely accepted that positive biodiversity effects increase with biotope space (Dimitrakopoulos and Schmid 2004). Biological mechanisms investigated in small-scale studies often have indicative character for processes at larger scales (van der Heijden et al. 2006), although the spatial and temporal scale of investigations and the researcher's control over experimental conditions (species pool, density of community, type of substrate, nutrient supply, duration of study etc.) often determines observable patterns and processes (Wiens 1989; Mikola et al. 2002; Hobbs and Norton 2004; Ejrnaes et al. 2006).

Biological mechanisms of plant-plant interactions are very hard to identify directly in nature or in (semi-)natural, large-scale experiments (which either mimics regional grassland habitats in relation to species pool and composition or use experimental plots in naturally grown communities). The determination of small-scale interaction processes, which often form the basis for observation of large-scale patterns, requires a high degree of researcher's control over the system, which is only possible in micro- and mesocosm studies. Thus, I conducted

studies under different environmental conditions and at different spatial scales to test the potential to transfer ecological theories derived from ecosystem level to smaller units like community, population or individual level (combination of insurance and stress gradient hypothesis: **manuscript 1**, **manuscript 2**, stress gradient hypothesis: **manuscript 3**, **manuscript 4**, **supplementary material**). The confirmation of ecological theories in micro- and mesocosm experiments could open the gates to test the outcome of large-scale manipulations (e.g. for restoration projects or the production of biofuels in natural habitats) on a much smaller scale with positive effects on cost-benefit calculations.

METHODS TO INVESTIGATE PLANT-PLANT INTERACTIONS

Shearer & Kohl (1986) reviewed methods to study the degree of biological nitrogen fixation (BNF) of atmospheric N₂ in legume species under natural conditions. The approximate range of BNF quantifies the potential of a legume species to act as N-donor for neighbouring receiver species. In a nutshell, four methods are available: (i) the nitrogen accumulation method, a method based on a comparison of N-accumulation in yield between N-fixing and non-fixing crops, (ii) the acetylene reduction essay, a method which uses nodulated roots to detect the presence of nitrogenase activity (the enzyme which is responsible for BNF) and measures the reduction of acetylene to ethylene per unit time per unit mass of nodule, (iii) methods based on the use of ¹⁵N enriched materials (tracer/label studies), which use ¹⁵N enriched N₂-gas, fertilisers, biological materials or solutions which are applied to the atmosphere (closed system), the soil (isotope dilution method) or directly to the plant (leaf/plant label methods; details see **manuscript 4**) and (iv) the $\delta^{15}\text{N}$ natural abundance method, which uses the ratio of the heavier over the lighter N-isotope (¹⁵N/¹⁴N) in a sample and a standard (air) to gain information about the N-source of a species (details see **manuscript 3**).

All methods provide advantages and disadvantages but especially the isotope dilution method, which has the potential to highlight the fate of a ¹⁵N-tracer through a whole system, found wide-spread application to study legume effects and N-transfer in agricultural settings (Chalk 1991; Høgh-Jensen and Schjøerring 1997; Gardner and Drinkwater 2009). Tracers are also used in grassland systems to study N-dynamics under natural conditions (Buchmann et al. 1992; Kahmen et al. 2006; Kahmen et al. 2008; Robson et al. 2010) or in relation to disturbance (see **manuscript 4**). The $\delta^{15}\text{N}$ natural abundance value of a sample is *per se* a function of the $\delta^{15}\text{N}$ values of its N-sources (Handley and Raven 1992) and acts as an

integrator of N-dynamics in a system (Robinson 2001). The method is less often used in agricultural studies (Bolger et al. 1995; Eriksen and Hogh-Jensen 1998; Moyer-Henry et al. 2006) but has some advantages for ecological investigations. It does not require any experimental treatments and it can provide information about the tightness of the N-cycle – and thus N-limitation (see manuscript 3 and Schulze et al. 1994; Nadelhoffer et al. 1996; Amundson et al. 2003; Pardo et al. 2006). Furthermore, $\delta^{15}\text{N}$ values can provide evidence for symbiotic relationships with different types of mycorrhizal fungi or N-fixing organisms as reviewed by Högberg (1997) and Dawson (2002). The $\delta^{15}\text{N}$ signal can provide information about N-transfer from donor to receiver species in biodiversity grassland experiments (Mulder et al. 2002; Spehn et al. 2002; Temperton et al. 2007; Carlsson et al. 2009) and in natural settings (Bai et al. 2009), but heterogeneity of natural plant communities with often high legume species presence and lack of adequate control plants, sets some limits to its applicability to study N-transfer in the field (see manuscript 3 and Handley and Scrimgeour 1997; Beyschlag et al. 2009).

Analyses of ^{15}N (^{15}N -tracer or $\delta^{15}\text{N}$ natural abundance) in plant and soil samples, although powerful tools to highlight interaction processes between legume donor and non-legume receiver species, have one major disadvantage: they require destructive sampling. Thus, every sampling disturbs the system to a certain degree; e.g. the cutting of leaves or even whole individuals can alter plant-plant interactions (effects of simulated grazing on N-transfer between different species: see **manuscript 4**). The sampling of root and soil material can alter substrate structures or facilitate subsequent invasion by creation of empty space (Buckland et al. 2001; Buckley et al. 2007). Therefore the use of non-invasive methods to study ecosystem processes is desirable, e.g. measurements of the leaf area index (LAI) to extrapolate (stratified) community productivity can substitute biomass harvest (Daßler et al. 2008; Vojtech et al. 2008). Information about individual or species response to environmental stresses can be derived from the measurement of chlorophyll *a* fluorescence of plant leaves and thus can partly substitute e.g. laborious pigment content analyses. The quantification of chlorophyll *a* fluorescence of photosystem II by PAM-fluorometers (pulse-amplitude modulated photosynthesis yield analyzers by H. Walz GmbH, Effeltrich, Germany) is a quick and non-invasive way of measuring the efficiency of light reactions *in situ* (Schreiber et al. 1986; Maxwell and Johnson 2000). Fluorescence measurements can indicate photosynthetic constraints due to drought stress (Rascher et al. 2004) or flooding (Pociecha et al. 2008). Still unsolved are the questions (i) if it provides a useful tool to detect changes in plant-plant interactions in the context of varying species richness along environmental gradients and (ii)

if it might provide a tool to predict productivity reductions due to environmental stresses. The studies presented in **manuscript 1** and **manuscript 2** address questions concerning the changes in response parameters (chlorophyll *a* fluorescence, individual biomass production and others) of a common European grass species in relation to community composition and legume presence under extreme weather stress.

OBJECTIVE OF THE THESIS

The objective of this doctoral project was to elucidate mechanisms involved in positive plant-plant interactions (facilitation) in relation to diversity and identity of species in communities and to test ecological theories (stress gradient hypothesis, biodiversity-productivity relationship, insurance hypothesis) at different spatial scales. The guiding questions for all studies conducted during this PhD were: How do interactions change along biotic gradients (species and functional diversity)? How do they change along abiotic gradients (nutrient status, disturbances like weather stress or simulated grazing)? And how do they change with the spatial scale (controlled greenhouse or climate chamber compared to (semi-)natural field experiments)? I performed basic ecological research; results could be useful for the field of applied ecology, e.g. for restoration of degraded habitats or sustainable biofuel production. Results should help to bridge the gap between theory and practice; knowing of this gap and aiming to reduce its width is a major challenge in modern ecology (Temperton et al. 2004).

I focused on interaction processes between grassland species, especially between legume species (as N-donors) and neighbouring non-legume species (as N-receivers). Species from the functional group of N₂-fixing legumes often have strong positive effects on neighbouring or subsequent species by providing extra-nitrogen (N-facilitation), although mechanisms of N-facilitation are mostly unclear. Nitrogen is a limiting factor in most terrestrial ecosystems (Chapin 1991; Vitousek and Farrington 1997; Marschner 2002), thus interaction processes between N-donor and N-receiver species play a key role for N-dynamics and productivity in (semi-)natural habitats (e.g. Spehn et al. 2002; van der Heijden et al. 2006; Temperton et al. 2007; Haultier et al. 2009) and in agricultural ecosystems (e.g. Giller and Cadisch 1995; Ledgard et al. 2001; Hogh-Jensen 2006; Moyer-Henry et al. 2006). N-facilitation is most important under N-limited conditions (Ledgard et al. 2001; Weigelt et al. 2009), but legume presence can easily shift to competitive pressure if other resources are limiting (Pugnaire and Luque 2001; Kikvidze et al. 2006; Haultier et al. 2009). Thus, the identification of a threshold where facilitation changes to competition and *vice versa* is of high interest. Although a vast amount of information about interaction processes between legume and non-legume species is already available, most studies on N-facilitation for receiver species have been conducted in species-poor, relatively eutrophic agricultural settings. Resulting main research questions, linking individual projects within this PhD, were:

- (i) Is it possible to investigate, by the use of short-term, small-scale experiments under more controlled conditions, the mechanisms behind positive plant-plant

interactions which are observed in long-term, large-scale studies under (semi-) natural conditions? Is it possible to simulate nature and to test ecological theories at (temporal and spatial) small scales and thus provide a tool to enhance the predictability of large-scale changes (due to land use or climate change)?

- (ii) Is it possible to identify a threshold where facilitation shifts to competition (testing the stress gradient hypothesis)?
- (iii) How do plant-plant interaction processes change in more diverse systems compared to species-poor systems at different scales (testing biodiversity-ecosystem functioning relationships) and how do changes in biodiversity affect species-specific responses of non-legume receiver species?

I used classical ecological census techniques (cover, biomass determination) but also more elaborated methods such as chlorophyll *a* fluorescence measurements and ¹⁵N-analyses to investigate legume effect on neighbouring receiver species under different environmental (both biotic and abiotic) conditions.

SYNOPSIS

OUTLINE OF MANUSCRIPTS

Main topic of this thesis was to elucidate mechanisms of positive and negative plant-plant interactions between grassland species at different spatial scales. In particular, I am interested in legume-neighbour interactions and how they change with diversity, species composition and identity along gradients of environmental stress and with the spatial scale of the study. I worked in small-scale microcosm experiments and in field settings to evaluate positive legume effects (N-facilitation) and to test the reproducibility of ecological theories across spatial scales. I used different invasive and non-invasive methods to investigate presence and strength of N-facilitation. The research had a pronounced focus on neighbours as N-receivers and not on the legumes themselves, acting as N-donors. The first two manuscripts describe studies that link the fields of biodiversity and climate change research; investigating which differences occur between monocultures and more diverse communities under the threat of altered climatic conditions. The second two manuscripts describe studies about legume-neighbour interactions that link the fields of restoration ecology and agriculture; considering how land-use changes may affect the interplay between species.

Within the study presented in **manuscript 1**, we investigated how a single target species (*Holcus lanatus*) performed under two environmental stresses (extreme drought and heavy rain events) in the context of varying diversity of the surrounding plant communities (G1:- monocultures, G2:- 2-species-mixtures, G4:- 4-species-mixtures without a legume species, G4+: 4-species-mixtures with the legume species *Lotus corniculatus*). In 2007, we measured photochemical efficiency (chlorophyll *a* fluorescence) and individual biomass production (NP_{ind}) of *H. lanatus* within the EVENT-Experiment I, located in the Ecological-Botanical Garden at the University of Bayreuth. We found, that chlorophyll *a* fluorescence of *H. lanatus* was only a poor predictor for NP_{ind} although it was a useful tool to detect drought stress in the target species whereas it failed to detect constraints related to the heavy rain treatment, which led to reductions in NP_{ind} (but not in the photochemical efficiency). Contrary to our expectations, drought effects on photochemical efficiency and NP_{ind} of *H. lanatus* were not detectable for monocultures but increased with increasing functional diversity in mixtures. At community level, negative effects on the target species were ameliorated by the performance of neighbouring species as reported in **manuscript 1** for the photochemical response and in Kreyling et al. (2008a) for total biomass production of communities. Especially the legume

species *Lotus corniculatus*, which used the available light resources very efficiently, affected the photochemical community response in G4+ and added significantly to the stability of the community. In **manuscript 1** we conclude, that negative effects of extreme drought on NP_{ind} and photochemical efficiency were mainly related to a decrease in competitive strength of *H. lanatus* (for soil water resources) in more diverse communities which led to an earlier senescence of the target species. Based on these findings, we performed a second study (presented in **manuscript 2**) to investigate the physiological response of *H. lanatus* to extreme drought stress in more detail.

The study presented in **manuscript 2** took place in the EVENT-Experiment I in 2008. We focused on the same target species but investigated its performance not only in terms of NP_{ind} and light- as well as dark-adapted photochemical efficiency but also by measurements of leaf water potential (LWP), N-parameters (N-concentration and $\delta^{15}\text{N}$ natural abundance) and photosynthetic pigment contents of *Holcus lanatus* leaves *within* and *after* the drought treatment period. It was possible to confirm the negative effects of increasing functional diversity (and especially of legume presence) on photochemical efficiency and NP_{ind} in *H. lanatus*, which points towards a general mechanism behind the findings. LWP of the target species was lowest in 4-species-communities with a legume species (G4+) under drought stress confirming a reduction of competitive strength in *H. lanatus* for limited soil water resources. On the other hand, it was not possible to show earlier senescence (accompanied by photosynthetic pigment degradation) due to drought stress as concluded from the first study. No significant differences in total photosynthetic pigment content occurred for *H. lanatus* along the diversity gradient or between control and drought treatments although a trend to decreased total pigment content was observed in both four species communities under drought stress. Most impressive was the fast and total recovery (within one week) of formerly drought stressed *H. lanatus* plants in G4+ in the post-drought phase: photosynthetic efficiency of light- and dark-adapted leaves showed a fast and complete recovery whereas monocultures, which were more stable *during* drought, still showed significant reductions in photosynthetic efficiency. The high degree of recovery compared to all other communities was considered as a clear sign of facilitation from the legume species *L. corniculatus*, measured by changes in N-concentration and $\delta^{15}\text{N}$ values, and provides evidence for higher stability and resilience in communities with higher functional diversity and thus the insurance hypothesis.

The study presented in **manuscript 3** was conducted to detect potential facilitative legume effects on the nitrogen metabolism of neighbouring non-legume species (receivers). We used an environmental N-gradient, provided by a large-scale calcareous grassland restoration project, to follow changes in donor-receiver interactions. The restoration site with its different treatments provided an ideal testing ground for the stress gradient hypothesis (related to N-facilitation): the differently treated areas are very distinct in terms of N-availability and N-forms in the soil solution, thus providing an N-gradient, but nevertheless in direct vicinity to each other, thus reducing confounding effects of e.g. climate. We aimed to show positive legume effects and increasing N-facilitation with increasing N-limitation in a (semi-)natural field site (according to the stress gradient hypothesis) using the $\delta^{15}\text{N}$ natural abundance method. The $\delta^{15}\text{N}$ signal in plants acts as an integrator of N-dynamics in a system but has also been successfully used to resolve N-facilitation of legume species for non-legume neighbours (Mulder et al. 2002; Temperton et al. 2007; Bai et al. 2009). We collected plant pairs and control plants along the N-gradient: donor-receiver pairs (legume species and non-legume neighbours) and control plants of the receiver species. Analyses of $\delta^{15}\text{N}$ values showed that all legume species had a constant $\delta^{15}\text{N}$ value along the N-gradient. Thus, legume species acted as potential N-donors and we expected highest N-facilitation at the most severe end of the N-gradient. Non-legume species showed a significant increase in $\delta^{15}\text{N}$ with decreasing environmental severity (from $\sim -7.5\text{‰}$ to $\sim 0\text{‰}$) with species-specific differences due to life form and mycorrhizal symbiosis of the species. In general, we found that $\delta^{15}\text{N}$ values were mostly under (abiotic and biotic) environmental control and provide only weak evidence for N-transfer from legumes to neighbours. Although the integrated signal from soil N-dynamics seemed to override any facilitative N-donor signal, the study revealed the potential of the $\delta^{15}\text{N}$ natural abundance method to indicate restoration success.

Manuscript 3 showed that the $\delta^{15}\text{N}$ natural abundance method might not always result in a detectable signal of N-facilitation by legumes. Thus, we performed a microcosm study to investigate, if it is possible to resolve small-scale differences in N-transfer with a ^{15}N -enriched tracer (**manuscript 4**). Aim of the study was (i) to compare short-term N-transfer within differently composed communities and (ii) to test the effect of a common management regime (grazing) on plant-plant interactions. We used communities of different compositions (one legume, grass and forb species in three diversity levels) and investigated effects of simulated grazing (cutting of aboveground biomass of the ^{15}N -labelled donor individual) on

^{15}N -transfer (measured as [%] of ^{15}N -tracer transferred from ^{15}N -labelled donor to non-labelled receiver individuals). We found a positive effect of species richness on ^{15}N -transfer: it increased significantly from monocultures to 3-species-mixtures, irrespective of community composition. A potential legume effect on ^{15}N -transfer was superimposed by a strong confounding effect of donor species biomass production. A significant positive legume effect, but no diversity effect *per se*, occurred on net biomass production per individual (NP_{ind}), N-concentration [%] and N-content ($= \text{NP}_{\text{ind}} \times \text{N} [\%]$). Interestingly, the grass species received significantly more ^{15}N from a legume donor than the forb species in 2-species-mixtures whereas in the 3-species-mixtures the amount of ^{15}N transferred from the legume was homogeneously distributed between grass and forb. Despite the same ^{15}N -enrichment in the grass and the forb species in 3-species-mixtures, the grass accumulated more NP_{ind} and had a higher total N-content than the forb, which indicated better nitrogen use efficiency of the grass species. Additionally, we found a highly interesting interaction between simulated grazing and species richness on ^{15}N -transfer: simulated grazing stimulated *intra-specific* N-transfer in monocultures whereas it reduced *inter-specific* N-transfer in mixtures. Contrary to our expectations, simulated grazing had (as a trend) an overall negative effect on ^{15}N -transfer and mainly increased internal N-cycling for regrowth of the cut individual. Thus, individuals seemed to “decide” how to organize their N-dynamics when grazed depending on the surrounding community; a finding that support recent publications about kin recognition and plant behaviour (*sensu* Karban 2008) and provide novel insights about the importance of community composition for plant behaviour.

SUMMARIZING CONCLUSIONS AND EMERGING RESEARCH QUESTIONS

The main strengths of this PhD research were twofold: firstly, the investigation of N-facilitation between legume donor and non-legume receiver species was extended from the traditional-agricultural two-species-interactions to a gradient of plant diversity, where the *diversity of communities* and the *identity of the interacting species* are crucial points for facilitative plant-plant interactions. Secondly, individual projects were conducted across a range of spatial scales and across (both diversity and) environmental gradients. This is important since main criticism of large-scale biodiversity field experiments has been that positive biodiversity effects, which have been found in such settings, may not be transferrable to other habitats and ecosystems or even to other grasslands (e.g. Kahmen et al. 2005, Guo 2007). We do not know to what extent effects found in semi-natural grassland experiments also apply at other scales; i.e. smaller or natural landscape scales (but see Kahmen et al. 2005, Kahmen et al. 2006 for rare landscape-scale studies). To find out more about the existence of biodiversity effects – and especially about positive legume effects (N-facilitation) – across scales and habitats, it is necessary to conduct research addressing the same questions in different habitats with varying environmental conditions and at different scales. Hence, I used systems from microcosms (pot experiments) up to macrocosms (field studies) to elucidate mechanisms of plant-plant interactions (mainly N-dynamics), and changes in these interactions in relation to the identity of species within differently composed communities. The combination of individual studies within my PhD project made it possible to *compare* effects of species composition and identity, legume presence and (to some extent) species richness *between* experiments, that represented a large variety of environmental conditions. Importantly, this allows for one to address the common criticism of large-scale biodiversity experiments (being only one example or one habitat), as well as addressing how biodiversity effects may differ when investigated at various scales and across gradients (although therefore this approach does not, of course, allow for detailed study of multiple aspects of each experimental system; see publications of the Jena Experiment and the EVENT-Experiments in Germany or the Cedar Creek Experiment in the USA for details hereof).

Results from this thesis provide novel insights into the ecology of temperate grassland systems. They are of interest for the field of biodiversity research (which has mainly been investigated by large-scale experimental set ups), for the field of facilitation research (which has mainly been investigated in natural and very extreme habitats and not in mesic habitats or experimental set ups) and for the fields of plant physiology and plant behaviour (which has mainly been investigated using single individuals and single species (i.e. autecology) with

little relevance for natural grassland systems). In the following, I outline and discuss the main findings of this PhD project in relation to the three main questions posed at the end of the Introduction.

TESTABILITY OF ECOLOGICAL THEORIES AT DIFFERENT SCALES

Ecological theories are derived from observations, which normally include effects of the biotic and the abiotic environment as well as interaction effects between organisms. Thus, testing the applicability of ecological theories, e.g. of the stress gradient hypothesis (SGH) for N-facilitation or of the insurance hypothesis, at different scales is not an easy task but well-designed studies have the potential to identify general patterns. Within this thesis I present studies, which showed that some of the mechanisms affecting plant population and community performance at field scale, also apply at the microcosm scale and thus, that it is indeed possible to test ecological theories at smaller scales.

Results presented within **manuscript 4** and **supplementary material** provide evidence for the testability of ecological theories within microcosms: we were able to confirm the environmental control over total community productivity (Cardinale et al. 2009; Huston et al. 2000) and the decrease of $\delta^{15}\text{N}$ values with increasing N-limitation and environmental severity, which were found in the field (e.g. Amundson et al. 2003; Pardo et al. 2006 and **manuscript 3**) as well as in a greenhouse experiment (with different diverse plant communities grown in substrates of low, medium and high N-availabilities; see **supplementary material**). Lower or even negative $\delta^{15}\text{N}$ values are related to a better N-conservation and N-recycling in colder, wetter and stronger N-limited systems, thus to a more closed N-cycle (e.g. Amundson et al. 2003; Pardo et al. 2006). We were able to show that these effects occur irrespective of the spatial scale of the studies (at least for the studies conducted within this PhD project).

This finding include that it is allowed to test the SGH at different scales within the temperate regions. There is evidence from either highly fertilised or unfertilised mesic (semi-)natural grassland habitats (Ledgard et al. 2001; Marquard et al. 2009a; Weigelt et al. 2009), that the importance of N-facilitation increases with N-limitation, as predicted by the SGH. Marquard et al. (2009a) show that positive biodiversity effects such as complementarity effects increased over time whereas sampling effects became less important during six years of the Jena Experiment. This is possibly due to the regular hay (and thus nutrient) removal after mowing accompanied by increasing N-limitation, which could also relate to an increase in facilitation

over time. However, we do not yet know how positive biodiversity effects interact with an environmental gradient of N-availability. To our knowledge, experiments with an N-gradient (from mesic to N-stressed conditions) in field or microcosm studies are generally rare.

The study presented in **supplementary material** suggests increasing N-facilitation with increasing N-limitation in the substrate and thus provides some support for increasing positive plant-plant interactions, according to the SGH, also at small scales. Here, N-facilitation occurred mainly by N-sparing and not by short-term N-transfer from donor to receiver species, indicated by higher N-concentrations in leaves of receiver species without homogeneous changes in $\delta^{15}\text{N}$ values. This finding is in accordance with effects found in the field: N-facilitation by N-sparing prevails in the short-term whereas N-transfer gets more important in the long-term (Hogh-Jensen and Schjoerring 2000, Temperton et al. 2007). The study presented in **manuscript 3**, conducted in a restored calcareous grassland (with four areas, which differ in their environmental N-availability), aimed to deliver some evidence for the SGH in terms of N-facilitation in a field setting within the temperate regions – but it seems that the integrative character of the $\delta^{15}\text{N}$ natural abundance method for the overall N-cycle excludes a detection of facilitative donor-receiver interactions under this natural conditions. On the other hand, increasing N-facilitation due to higher environmental stress might have had happened for donor-receiver pairs along N-gradients at large and small spatial scales (**manuscript 3, supplementary material**) and during extreme weather stress on an intermediate scale (**manuscript 1, manuscript 2**) but we were not able to *detect* it. Here, the application of multiple or simple stable isotope tracers may provide a more powerful tool than the $\delta^{15}\text{N}$ natural abundance method to investigate changes in N-facilitation along abiotic or biotic stress gradients. The study presented in **manuscript 4** provides evidence that ^{15}N -enriched substances have the potential to highlight changes in plant-plant interactions due to species diversity and species composition. Additionally, this study provides novel insights, elucidated by a ^{15}N -tracer, on interactions between community compositional and disturbance effects for the young field of plant behaviour and kin recognition (*sensu* Karban 2008).

Concerning the testability of the insurance hypothesis by microcosm studies, the set up of an adequate design is even more crucial than for tests of the SGH, because the SGH mainly predicts the outcome of pair-wise interactions (Maestre et al. 2009) whereas the insurance hypothesis predicts the outcome of whole communities – although the community response depends on species responses (Yachi and Loreau 1999). Thus, the extrapolation from species responses to higher organisation levels (e.g. communities) must be done with extreme caution

because the same factor often affects different scales and organisation levels very differently (Balvanera et al. 2006). The studies on the stress response of *Holcus lanatus* under semi-natural field conditions (**manuscript 1**, **manuscript 2**) clearly showed, that species- and community-specific responses can differ significantly: whereas the applied stresses had significantly negative effects on species level, the effects on community level were only marginal. We were able to show, to our knowledge for the first time, that a non-invasive method to measure the physiological performance (chlorophyll *a* fluorescence) of *individuals* from different grassland species also provides a promising tool to investigate *community* responses (**manuscript 1**). The question arises, if it might be possible to use a mixture of non-invasive methods (fluorescence and leaf area index (LAI) measurements) to substitute invasive methods (harvest, element analyses in tissues) for the investigation of community responses to environmental stresses, e.g. to test the insurance hypothesis in natural communities. The use of non-invasive methods provides the possibility to study community responses to a treatment repeatedly and without confounding treatment effects by additional disturbances of the system due to e.g. harvest.

TESTABILITY OF BIODIVERSITY AND LEGUME EFFECTS AT DIFFERENT SCALES

Asking the question, if it is possible to simulate plant-plant interaction effects, which have been observed in nature or in large-scale, long-term ecological experiments (field-effects), at smaller scales (and also *vice versa*!), no absolute positive or negative answer can be provided because both seems to be true. Spatial scale is an indisputable factor for the outcome of an observation (Balvanera et al. 2006; Dimitrakopoulos and Schmid 2004; Sandel and Smith 2009) but this fact does not exclude the use of smaller units (e.g. populations) to predict biodiversity or legume effects in larger units (e.g. habitats). The studies presented within this thesis led to the conclusion, that the use of experiments on a relative small spatial scale under more controlled conditions can provide important information for the prediction of species interactions due to large-scale manipulations. Thus, we provide additional support for the view, that small-scale experiments indeed have an indicative character for processes observable at larger scales as stated by van der Heijden et al. (2006), especially related to N-dynamics. This finding opens a new application spectrum for studies within controlled environments (greenhouse, climate chamber) for the research of N-dynamics under changing abiotic (N-availability, disturbance, management regime) and biotic (species diversity, composition and identity) conditions. Especially the investigation of *interactions between abiotic*

and biotic factors, and their changes with changing environmental severity or management regime, is possible in greenhouse studies, whereas both factors are nearly impossible to separate in the field. Additionally, a separation of real “biodiversity effects” and “compositional/sampling effects” is much easier if highly replicated micro- or mesocosm studies could be used. For example, patterns of legume-neighbour interactions on N-dynamics can be elucidated, *if* performed under comparable environmental (both abiotic and biotic) conditions - at least for early successional communities or for plant-plant interactions after disturbance. Investigations of biodiversity or compositional effects at small scales must be clearly focussed on a certain function or process (e.g. changes in N-cycling) and generalizations to other functions or processes (e.g. community biomass production) should be avoided.

We found positive *per se* biodiversity effects on N-parameters in a microcosm study (**manuscript 4, supplementary material**; stronger under mesic than under low N-conditions) and in a mesocosm study (**manuscript 2**; stronger under drought than under ambient conditions). Individual N-concentrations and $\delta^{15}\text{N}$ values decreased with increasing species richness of the surrounding community as it has been observed in large-scale field experiments (Mulder et al. 2002; Temperton et al. 2007), providing first experimental evidence, that biodiversity effects on N-dynamics are comparable between small- and large-scale experiments, opening the opportunity to study the mechanisms behind this phenomena under controlled conditions. On the other hand, we also could confirm the occasionally strong occurrence of key species effects within microcosm studies; e.g. the sampling effect of legume species presence on community biomass production (**manuscript 4** and Mikola et al. 2002; Spaekova and Leps 2001). Higher probability for the occurrence of sampling effects are related to the fact that experimental and natural systems generally differ strongly in their community assembly (Ejrnaes et al. 2006; Hobbs and Norton 2004) and that potential founder effects can affect the outcome of a study significantly (Körner et al. 2008; Šmilauerová and Šmilauer 2010). Additionally, higher asymmetric competition occurs between species under controlled than under field conditions (Ejrnaes et al. 2006; Hobbs and Norton 2004). Due to an increased importance of the sampling effect in smaller experimental units, a positive effect of biodiversity *per se* on community productivity, which is evident in field experiments (Roscher et al. 2005; Marquard et al. 2009a; van Ruijven and Berendse 2009), could be simulated only on some occasions in the greenhouse (Lanta and Leps 2006 and **supplementary material**). A kind of sampling effect was also evident for the photosynthetic efficiency (and biomass production) of *Lotus corniculatus* within the EVENT-Experiment I (**manuscript 1**). A highly interesting future research would be, to identify the contribution of *L. corniculatus* to comm-

unity's biomass production, photosynthetic efficiency and its potential to act as an N-donor in microcosms, the EVENT-Experiment II (which comprises of naturally grown grassland natural communities) and in natural meadows.

Positive effects of legume presence on N-availability for neighbouring non-legume species have been found in (semi-)natural grassland habitats as well as in micro- and mesocosm experiments (Ledgard et al. 2001; Mulder et al. 2002; Temperton et al. 2007 and **manuscript 2, manuscript 4, supplementary material**). Especially grass species benefited from an N-donor in the community both in field and in microcosm experiments. Although N-sparing is more important than N-transfer in the short-term (**manuscript 4, supplementary material** and Temperton et al. 2007), N-transfer *does* occur during a 20-30 days period (**manuscript 4** and Gylfadottir et al. 2007). Grasses received more N by direct N-transfer and competed effectively with forb species for limited soil N-resources during two three-month microcosm studies (**manuscript 4, supplementary material**). This short-term observations reflect processes which occur in (semi-)natural communities (Mulder et al. 2002; Jumpponen et al. 2005; Oelmann et al. 2007; Temperton et al. 2007), providing further evidence for the testability of biodiversity and legume effects on non-legume receiver species at small scales. Different studies already show that patterns of N-uptake vary between different functional groups and species (Weigelt et al. 2005; Kahmen et al. 2006; von Felten et al. 2008) but it would be very interesting to elucidate the mechanisms behind this findings, especially embedded within a biodiversity context, and if mechanisms are the same on different spatial scales. Furthermore, it would be interesting to study the N-acquisition strategy of stress-adapted species and if such grasses exhibit the same advantage in N-acquisition over forbs when growing together with a legume neighbour as mesic-adapted grass species. Until now, most studies use species, which are adapted to mesic or eutrophic conditions due to their relevance for pasture production.

We also could confirm N-transfer from non-legume species to neighbours within microcosms with a natural substrate (**manuscript 4**); this bi-directional N-transfer has been described for the field as well as for highly artificial lab settings (Hogh-Jensen 2006; Hogh-Jensen and Schjoerring 2000; Paynel and Cliquet 2003). A next step to test biodiversity, compositional and legume effect in microcosm should be to increase the diversity and test more species pairs and more community compositions for the generalisation of finding; not only across spatial but also across temporal and environmental gradients. The temporal gradient is highly inter-

esting because e.g. Marquard et al. (2009a) find increasing importance for complementarity and decreasing importance of sampling effects over six years.

BIODIVERSITY AND LEGUME EFFECTS ALONG ENVIRONMENTAL GRADIENTS

Asking the question if it is possible to identify a threshold in legume-neighbour interactions where facilitation shifts to competition along an abiotic gradient and how the surrounding species richness modulates the interaction processes, no clear answers could be provided. In general, legume effects are less pronounced under high N conditions but they can replace high fertiliser application rates in mesic habitats (Ledgard et al. 2001; Weigelt et al. 2009). Legumes are considered to be relatively poor competitors for soil N-resources because of a less extensive root system (e.g. Craine et al. 2002) and in combination with the fact, that nearly all natural habitats are N-limited (differing mostly in the degree of N-limitation) legume species are often forced to rely on biological nitrogen fixation (BNF) to sustain their N-demand. Thus, legume species are generally a potential N-source for neighbouring species. We were able to identify some interesting patterns in relation to thresholds under mesic conditions: firstly, that the diversity of surrounding non-legume species seemed to determine the legume's use of soil N-resources and secondly, that the benefits for receiver species from N-facilitation were stronger than short-term effects of space competition.

Along biotic gradients, more niches are occupied at the more diverse end of the gradient and total N-exploitation of a community increases (Oelmann et al. 2007; Palmborg et al. 2005); an effect which is reflected in decreasing $\delta^{15}\text{N}$ values with increasing species richness irrespectively of legume presence in the field as well as under low and medium N-availability in a greenhouse study (Mulder et al. 2002; Temperton et al. 2007 and **supplementary material**). Positive legume effects should increase in more diverse communities because of a more closed N-cycle. We found some support for a changed N-acquisition strategy in legume species under medium N-availability with increasing diversity of the surrounding community (**supplementary material**). Whereas legumes' $\delta^{15}\text{N}$ values were positive (acquisition of N from soil) when it grew with two neighbours, $\delta^{15}\text{N}$ values switched to negative (acquisition of N from BNF) when legumes grew with three neighbours. The change may reflect increasing competition for N-resources with increasing species richness and also a kind of start-off state for N-facilitation for neighbouring non-legume species. Additionally, results from a second microcosm experiment (**manuscript 4**) pointed in the direction of interacting effects of

diversity and legume presence for N-facilitation along a biotic gradient. We did not observed higher competition for rooting space or light between non-legume and legume species despite the vigorous growth of the legume species. On the contrary, vigorously growing legume donors enhanced biomass and N-accumulation in receivers compared to smaller non-legume donors and thus we detected an increase in N-facilitation with diversity but no changes in competition within this microcosm study in medium N-supplied substrate. This very interesting interplay between species richness and potential N-facilitation *within* a constant mesic environment still requires more research, especially because Haultier et al. (2009) identified space as the main limiting resource under mesic to eutrophic conditions. Further work under controlled and field conditions, with more diverse communities and within systems which mirror real habitats, e.g. in need of restoration, should help to fully understand changing donor-receiver interactions along biotic gradients.

Concerning abiotic gradients, we could show that the $\delta^{15}\text{N}$ natural abundance method was a better indicator for soil N-dynamics than for N-facilitation (**manuscript 3**). Low $\delta^{15}\text{N}$ signals in potential receivers indicate a more closed N-cycle (and thus a potential higher *requirement* for N-facilitation). Although we found that the $\delta^{15}\text{N}$ natural abundance method was not appropriate to investigate N-facilitation in very nutrient depleted calcareous grasslands we did find highly interesting, that there is the strong potential to use the $\delta^{15}\text{N}$ signal in non-legume grassland species to assess N-dynamics in the soil (e.g. in ecological restoration projects). Thus, the $\delta^{15}\text{N}$ signal in plants may substitute laborious soil N analyses, whose results are strongly affected by season and climate. Recent work e.g. by Kahmen et al. (2008) corroborate the indicative character of the $\delta^{15}\text{N}$ natural abundance method for soil N-processes. Plans for further work imply a “screening” of natural systems with different degrees of N-limitation and the addition of ^{15}N -enriched tracers, which may highlight increasing N-facilitation with increasing N-limitation. Here, collaborations with specialists in the field of mycorrhizal fungi, microbiology or soil chemistry could contribute to separate effects of the abiotic environment from effects related to interactions between lower and higher biological organisation forms.

Along abiotic gradients, the identification of thresholds where N-facilitation switches to competition is also important if a second resource besides N limits plant growth. Legume species often compete with other species for available resources (water, phosphorus, light, space) and thus, can have negative effects on neighbouring species – but when and how a switch occurs is, to our knowledge, still mainly unknown. Results from the studies presented

in **manuscript 1** and **manuscript 2** indicate that under drought stress, the legume *Lotus corniculatus* was very effective in competing for soil water resources with neighbouring species. This affected photosynthetic performance and water potential of the neighbouring grass species *Holcus lanatus* significantly negative during an extreme drought event. Interestingly, in the rewetting phase *after* drought, legume presence had a positive effect on the recovery of *H. lanatus*. On the other hand, under persistent undisturbed (ambient) conditions we found no explicit facilitative effect of legume species presence or higher diversity on photosynthetic efficiency or pigment content in leaves of *H. lanatus* although higher N-concentrations and $\delta^{15}\text{N}$ natural abundance values indicate N-facilitation under ambient conditions (**manuscript 2**). Thus, we provide first evidence for a switch from N-facilitation to competition for soil water resources under short-term extreme drought events within a mesic grassland habitat. This phenomenon is worth a more detailed investigation, especially in combination with the higher recovery potential of *H. lanatus* in the post-drought phase with than without a legume neighbour. Additionally, the study of other species in such a detail would result in valuable information about the general mechanism behind this (mediated) stress response. Again, the additional application of stable isotope tracers to plants or soils would certainly yield results which help to explain the described patterns.

CONCLUSION

In conclusion, I am confident that the findings presented within this thesis help to expand scientific knowledge on plant-plant interactions and how they relate to species identity and plant diversity. I provide novel insights (i) on the potential to use non-invasive methods for describing individual and community response to stresses, (ii) on the potential to use established methods like the $\delta^{15}\text{N}$ natural abundance method under a new point of view (namely as an indicator than as an integrator) and (iii) about plant-plant interactions and plant behaviour (*sensu* Karban 2008) and performance within differently composed communities under undisturbed or disturbed conditions. Results about underlying mechanisms and changes in these mechanisms with changing community diversity (mainly focussed on N-dynamics, especially between N_2 -fixing legume species and non-fixing neighbours) gained by small-scale, short-term or medium-term greenhouse studies, can have considerable implications at larger spatial and temporal scales and can help to explain patterns described by ecological theories. I am convinced that the study of plant-plant interactions and the knowledge of how such interactions change under different abiotic and biotic environmental conditions can help

to solve problems in a number of fields such as sustainable extensive agriculture (where legumes as fertilisers will play a larger role with increasing mineral fertiliser prices), biofuel production or the successful restoration of degraded habitats.

LIST OF MANUSCRIPTS AND DECLARATION OF OWN CONTRIBUTION

OWN CONTRIBUTION

Different sections of “own contribution” describe the process from initialisation of the study until the completion of the final version of each manuscript. I present only my own contributions because listing of every author’s contribution for multi-author manuscripts would go far beyond the scope of a short description.

Single sections comprise:

concept = idea for the study and development of the experimental design

data acquisition = taking care and responsibility for survival of microcosms and for measurements in the field

data analysis = translation of raw data in digital tables, statistical analyses

literature research = acquisition of background information for introductions and discussions

writing = translating all the words concerning a study from the brain to the computer by the use of my hands and a computer

discussion = integration of results in the context of the latest scientific state-of-the-art

editing = rewriting after discussion and implementation of improving comments for final versions of manuscripts

MANUSCRIPT 1

Title: *Holcus lanatus* under climate change stress – impacts of plant diversity and simulated extreme weather events on photosynthetic performance and productivity

Authors: Lea L.A. Martin, Vicky M. Temperton, Kerstin Grant, Julia Walter, Jürgen Kreyling, Carl Beierkuhnlein, Ulrich Schurr, Uwe Rascher, Anke Jentsch

Corresponding author: Uwe Rascher, Vicky Temperton

Status: submitted

Journal: Oecologia

Own contribution: concept (10 %), data acquisition (100 %), data analyses (70 %), literature research (70 %), writing (70 %), discussion (60 %) and editing (60 %)

MANUSCRIPT 2

Title: Presence of a legume species reduces the ecophysiological performance of *Holcus lanatus* during a drought, but speeds up recovery after drought stress

Authors: Julia Walter, Uwe Rascher, Lea L.A. Martin, André Moersch, Maik Veste, Carl Beierkuhnlein, Matthias Gehre, Anke Jentsch

Corresponding author: Julia Walter

Status: submitted

Journal: Environmental and Experimental Botany

Own contribution: concept (20 %), data acquisition (0 %), data analyses (20 %), literature research (5 %), writing (10 %), discussion (20 %) and editing (30 %)

MANUSCRIPT 3

Title: The use of the $\delta^{15}\text{N}$ natural abundance method to assess facilitation and restoration success in calcareous grassland

Authors: Lea L.A. Martin, Kathrin Kiehl, Daniela Röder, Andreas Lücke, Vicky M. Temperton

Corresponding author: Lea Martin

Status: in preparation for re-submission

Journal: Restoration Ecology

Own contribution: concept (50 %), data acquisition (100 %), data analyses (95 %), literature research (95 %), writing (95 %), discussion (70 %) and editing (90 %)

MANUSCRIPT 4

Title: N-transfer between grassland species: effects of community composition, species identity and simulated grazing

Authors: Lea L.A. Martin, Uwe Rascher, Ulrich Schurr, Vicky M. Temperton

Corresponding author: Lea Martin

Status: submitted

Journal: Functional Ecology

Own contribution: concept (75 %), data acquisition (100 %), data analyses (100 %), literature research (100 %), writing (90 %), discussion (70 %) and editing (80 %)

CONFERENCES AND TRAININGS

When	Where	Theme	Talk	presentation time [min]	Poster	Titel
05.-09.02.2007	Wageningen, NL	summer school in Soil Ecology: Crossing the frontier between below- and above-ground	x	10 + 5		Positive and negative dynamics of plant-plant interaction and their functional role in regulation ecosystem processes - with respect to the soil carrying the population
15.-19.09.2008	Leipzig, D	GfÖ-EURECO-conference 2008: Biodiversity in an Ecosystem Context			x	Positive biodiversity and legume effects - how relevant are they along a gradient of nutrient availability? (about FCE-experiments in pots, greenhouse)
05.12.2008	Bayreuth, D	Biogeographie-Lehrstuhlkolloquium	x	30 + 15		Positive and negative dynamics of plant-plant interactions and their functional role in regulating ecosystem processes (Vorstellung meines Promotionsthemas)
02.04.2009	Bayreuth, D	BayCEER Workshop 2009			x	Plant-plant interactions along biotic and abiotic gradients
20.-22.04.2009	Aberdeen, UK	BES Symposium 2009: Facilitation in Plant Communities			x	The use of stable isotope natural abundance to assess facilitation and restoration success in a calcareous grassland (about Garchinger Heide 15N-data)
29.06.-03.07.2009	Münster, D	SER summer school: Species introduction and management of biodiversity in restoration projects	x	7 + 3		Vorstellung meines Promotionsthemas: positive and negative dynamics of plant-plant interactions and their functional role in regulating ecosystem processes - mainly Garchinger Heide study
14.-18.09.2009	Bayreuth, D	GfÖ-conference 2009: Dimensions of ecology - From global change to molecular ecology	x	10 + 2		The use of d15N natural abundance to assess facilitation and restoration success in a calcareous grassland
27.11.2009	Düsseldorf, D	“Von der Idee zum Projekt – Finanzierung von Forschungsprojekten durch Drittmittel”				
15.04.2010	Bayreuth, D	BayCEER Workshop 2010	x	10 + 2		N-transfer between species: effects of legume presence and simulated grazing

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MANUSCRIPT 1

HOLCUS LANATUS UNDER CLIMATE CHANGE STRESS – IMPACTS OF PLANT DIVERSITY AND SIMULATED EXTREME WEATHER EVENTS ON PHOTOSYNTHETIC PERFORMANCE AND PRODUCTIVITY

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ABSTRACT

An increase in extreme precipitation events and loss of biodiversity associated with global change demand deeper insights into species performance under such disturbances. We investigated effects of extreme weather events on *Holcus lanatus* in the context of varying diversity in a field experiment.

We investigated the performance of *H. lanatus* in four diversity levels under extreme drought, heavy rain and in an ambient control by measuring non-invasive chlorophyll *a* fluorescence and compared the results with an invasive biomass harvest. Photosynthetic responses were also measured for neighbouring species in more diverse communities.

On species level (*H. lanatus*), we found strong negative effects of drought on maximum electron transport rate (ETR_{max}) and biomass production per individual (NP_{ind}) as well as a decrease in NP_{ind} in the heavy rain treatment. On community level (all species) ETR_{max} were not affected by weather treatments but strongly by legume presence. Effects on *H. lanatus* were most pronounced in more diverse communities indicating competitive stress and resource limitation for the target species. Community responses support our conclusions. ETR_{max} and NP_{ind} of *H. lanatus* were not simply correlated but were influenced differently by biodiversity and weather manipulations. Our results do not support hypotheses of positive effects of increasing species richness on the performance of a single species (*H. lanatus* as a beneficiary); *H. lanatus* performed worse in higher diversity plots under environmental stress; also the overall community response was not affected. Results suggest that more species-specific investigations on interactive effects of diversity and climate change are needed.

Keywords

chlorophyll *a* fluorescence, disturbance, drought, EVENT-Experiment, photosynthesis

Abbreviation list

C	= ambient control for weather manipulations
R	= heavy rain weather manipulation
D	= extreme drought weather manipulation
G1-	= monoculture of <i>Holcus lanatus</i>
G2-	= 2 species mixture (2 grasses)
G4-	= 4 species mixture without a legume species (2 grasses, 2 forbs)

G4+	= 4 species mixture with a legume species (2 grasses, 1 forb, 1 legume)
PS II	= photosystem II
PFD [$\mu\text{mol m}^{-2} \text{s}^{-1}$]	= photon flux density; used here for light with a photosynthetic active wavelength range of $\lambda = 380\text{--}710 \text{ nm}$
$\Delta F/F_m'$	= effective quantum efficiency (yield) of PS II in light-adapted leaves
$F_v/F_m\text{expo}$	= extrapolated potential quantum efficiency (yield) of PS II of light-adapted leaves
ETR	= apparent rate of photosynthetic electron transport of PS II
ETR _{max}	= maximum ETR
NP _{ind}	= net biomass production of <i>H. lanatus</i> individuals [g/tussock]

INTRODUCTION

We are living in a world of accelerated global change, including climate change and biodiversity loss, such that it is becoming increasingly important to understand the interaction between effects of diversity loss and climate change on the performance of organisms. The frequency and magnitude of extreme weather events is predicted to increase during global climate change (IPCC 2007). Extremeness of events rather than the mean changes in temperature and precipitation are (in some cases) expected to have the largest effects on ecosystem functioning (Meehl et al. 2000). Extreme weather events, however, have not yet received much attention in vegetation-related climate change research (Jentsch et al. 2007). Furthermore, the few existing experimental studies on extreme weather events often lack details on the magnitude or extremeness of the applied manipulations relative to local mean conditions (Jentsch 2006) although recent studies in semi-natural grasslands have shown that indeed local conditions are important for the performance of plant communities under environmental stress (e.g. Gilgen and Buchmann 2009).

Drought and heavy rainfall are generally expected to affect plants via modification of soil moisture which affects nutrient availability and thus plant growth. Water shortage leads to a decline in water potential and to water stress. An excess of water in soil pores creates oxygen deficits and produces a chemically reducing environment in the soil (Marschner 2002). The lack of oxygen can cause substantial short-term fine root mortality, even though species reactions differ considerably (Crawford and Braendle 1996). Both drought and heavy rainfall can harm individual species or whole communities and the effects can vary from productivity reduction up to a complete collapse of local vegetation and its accompanying ecosystem services when exceeding critical magnitudes (Marschner 2002; IPCC 2007).

Effects of extreme weather events should theoretically be modulated by the diversity of plant communities (McNaughton 1977). We know that species rich communities are often more productive than less diverse communities, especially in experimental grasslands (e.g. Hector et al. 1999; Balvanera et al. 2006). Furthermore, according to the insurance hypothesis, in the face of disturbance, high diversity buffers ecosystems against species loss and concomitant decline in ecosystem functioning: a large number of species (each perhaps redundant under one set of environmental conditions yet critical for functioning under altered conditions) improves the chances that overall ecosystem functioning will be maintained under fluctuating environmental conditions (McNaughton 1977; Naeem et al. 1994; Naeem and Li 1997; Yachi and Loreau 1999). Thus, under disturbance, the species-rich community will be better

equipped to resist or be resilient to changes than the species-poor community (Tilman and Downing 1994; Yachi and Loreau 1999).

Two main sets of hypotheses are usually put forward to explain the positive relationship between biodiversity and ecosystem functioning: niche complementarity and facilitation or sampling/selection effects. Complementarity through niche partitioning is considered as a mechanism whereby the sum of all species in species-rich communities with a high variety of traits are more effectively able to use resources in the system than fewer species in less diverse communities (Berendse 1979; Tilman 1997). Facilitation, the ability of one species to modify the environment beneficially for another species (Connell and Slatyer 1977), is often seen as a sub-category of niche complementarity: one species has a positive effect on a second (neighbouring or subsequent) species by enhancing the realised niche of the second species (Bruno et al. 2003). The classic facilitation example in biodiversity grassland experiments is the beneficial effect of nitrogen-fixing legume species on the nitrogen dynamics of non-fixing neighbours with knock-on effects on the productivity of the whole community (e.g. Spehn et al. 2002; Temperton et al. 2007). Sampling or selection effects are artefacts of biodiversity experiments choosing species mixtures at random (with replacement) and describe the increasing likelihood of including very productive or keystone species (or functional groups) with increasing diversity of mixtures (Huston 1997).

Increased disturbance and physical stress level are thought to reduce the intensity of competition and to increase the importance of facilitation (e.g. Holmgren et al. 1997; Brooker and Callaghan 1998) as summarized in the stress gradient hypothesis (SGH, Bertness and Callaway 1994). Most research on the SGH has been done in intertidal marine or extreme arid or alpine terrestrial habitats (Brooker et al. 2008). Studies focussing on mesic grassland habitats within the temperate regions of Europe are scarce although even under benign environmental conditions extreme weather events might increase the dependence of individual species on facilitation. Callaway and Walker (1997) reviewed earlier literature on the balance between competition and facilitation as driver of community structure and recent studies support the conclusion that the importance of facilitation increases with decreasing availability of the limiting resource within European subalpine grasslands (Kikvidze et al. 2006; Gross et al. 2009). In addition, complementary resource use among different species or functional groups can ameliorate the stress experienced by plants under harsh conditions (Pugnaire and Luque 2001; Gross et al. 2007). The facilitative effect of a legume shrub on the performance of other species under its canopy was stronger at the more stressful end of an environmental gradient compared to the more moderate end (Pugnaire and Luque 2001) but

little is known about how changes in environmental stress affect legume-neighbour interactions for herbaceous species within mesic grasslands.

Effects of plant diversity are often different at the community level compared to effects on plant population (see Balvanera et al. 2006) or individual level (Daßler et al. 2008; Kreyling et al. 2008c). Thus, we need to investigate effects of diversity and disturbance on individual, population as well as on community level, preferable by an easy-to-use, quick and non-invasive method. In the present study, we focus on the species-specific response of one species, *Holcus lanatus* L. (Yorkshire fog, Poaceae) within a field experiment. We investigate how i) the simulation of extreme weather events (drought or heavy rain) and ii) the composition of surrounding plant communities modulates an instant measure (non-invasive chlorophyll *a* fluorescence) and an integrated measure (harvest of individual biomass) of *H. lanatus*. Additionally, we evaluate the response of the surrounding species to extreme weather events non-invasively to gain a community response and to compare the use of the instant measure on population vs. community level. The target species *H. lanatus* is common in mesic grasslands on a wide variety of soils across Europe and a good competitor in benign environments (Beddows 1961; Veresoglou and Fitter 1984; Coll et al. 2003; Wurst and van Beersum 2008). The species is known to be sensitive to severe drought conditions (Pedrol et al. 2000) and to flooding (Liem 1980) but is well adapted to mild physical disturbance (gap creation, Buckland et al. 2001). *H. lanatus* is able to utilize mineral N-forms as well as amino acids for its N-nutrition (Weigelt et al. 2003; Weigelt et al. 2005) and can buffer water stress by utilising moisture from fog (Corbin et al. 2005). Thus, *H. lanatus* should be an ideal candidate to test species interactions in relation to the stress gradient hypothesis (and partly the insurance hypothesis), because the “*facilitative outcome appears to be a function of a species having both a low tolerance to a particular abiotic stress and a strong competitive response ability*” (Liancourt et al. 2005). We are not aware of any other studies on mesic grassland species which investigate population and community responses within an outdoor, yet well-directly manipulated, semi-natural field experiment. Most results on this topic were either gained under more controlled greenhouse conditions (e.g. De Boeck et al. 2006; Zavalloni et al. 2009) or under natural conditions without any explicit weather treatments (e.g. Verheyen et al. 2008). Thus our study can provide an important link between research under controlled conditions on the one hand and natural conditions on the other hand.

We propose two hypotheses for the instant and integrated response of *H. lanatus* to extreme weather events and one for the overall community response, evaluated by a non-invasive instant measure:

- i. Severe weather stress (drought and heavy rain) effects on the target species *Holcus lanatus* will be detectable by an instant measure of the photosynthetic efficiency (chlorophyll *a* fluorescence), and reduced photosynthetic efficiency will lead to reduced productivity per individual (integrated measure) under extreme weather conditions.
- ii. The expected negative response of *Holcus lanatus* individuals to extreme drought, the major threat for this species, will be ameliorated in more diverse communities.
- iii. On community level, negative effects of drought and heavy rain on the photosynthetic response of *Holcus lanatus* will be ameliorated by increased performance of neighbouring species in functionally more diverse communities as predicted by the insurance hypothesis.

MATERIALS AND METHODS

The study was conducted in summer of 2007 in the grassland plots of the EVENT-Experiment at the University of Bayreuth, Germany (EVENT-Experiment 2005). *Holcus lanatus* was chosen as target species, because of its importance in European grasslands and its availability in nearly all factorial combinations in the experiment. The EVENT-Experiment is a field experiment carried out with a two-factorial design manipulating (1) weather events (drought, heavy rain, ambient control) and (2) functional plant diversity (Jentsch et al. 2007). It was set up in 2005 at the Ecological-Botanical Gardens of the University Bayreuth, Germany (49°55'19''N, 11°34'55''E, 365 m asl). Mean annual temperature is 7.8°C; mean annual precipitation is 709 mm, usually distributed bi-modally with the major peak in June/July and a second peak in December/January (Data: Deutscher Wetterdienst). To prevent confounding effects by soil properties, a homogenized substrate, made of loamy sand (82% sand, 13% silt, 5% clay), pH = 4.5 and 6.2 (measured (1M KCl) in upper and lower soil layer), was added to the site during the set up of the experiment. The weather manipulations, each replicated five times, were applied in a randomly distributed block design over the total area of the experiment with the experimental plant communities randomly embedded within these blocks (see Jentsch et al. 2007 for details). The grassland plots consist of five species typical of mesic *Molinio-Arrhenatheretea*-meadow communities (Pott 1995) grown at four diversity levels (monocultures (G1-), two species mixtures (G2-) and four species mixtures without (G4-) and with (G4+) a legume species). The sizes of the plots were 1x1 m for the monocultures and 2x2 m for the other diversity levels.

Factor 1: extreme weather events

Manipulations consisted of extreme drought, heavy rain and an ambient control. Magnitude of manipulations was chosen according to the local 100-year extreme event in each category. Reference periods were the vegetation periods of the local climate data set from 1961 to 2000 (March to September for each year; Data: Deutscher Wetterdienst). For this time period, Gumbel I distributions were fitted to the annual extremes, and 100-year recurrence events were calculated (Gumbel 1958). Maximum values in the historical data set were 33 days without rain (June and July 1976) and 152 mm rain within a period of 14 days (June 1977). Accordingly, a drought event of 32 days (drought manipulation = D) and a heavy rainfall event of 170 mm within a period of 14 days (heavy rain manipulation = R) were applied in the

experiment during the peak growing season in June from 2005 to 2007 (Fig. 1). A control (ambient natural conditions = C) was used to evaluate manipulation effects.

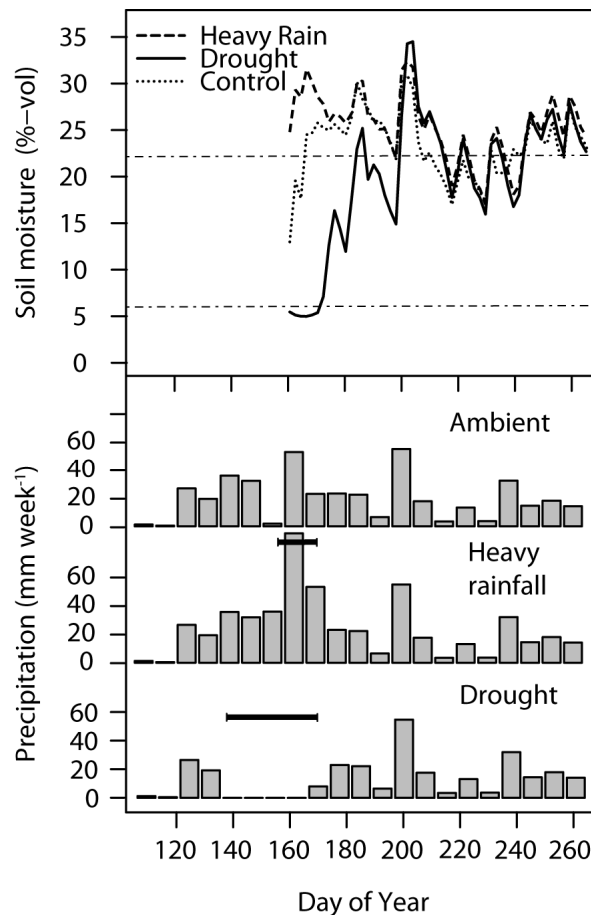


Figure 1 Soil moisture and precipitation in the EVENT-Experiment during manipulation and recovery after extreme drought and heavy rainfall events. 48 h mean values of hourly measurements by FD-sensors between -5 and -10 cm are given ($n = 5$ per weather manipulation). Upper dot-dashed line indicates field capacity ($pF = 1.8$), lower dot-dashed line indicates permanent wilting point ($pF = 4.2$) of the soil substrate (AG Boden 1996, Bodenkundliche Kartieranleitung). Grey bars show weekly precipitation for the weather manipulations in comparison with ambient conditions (control). Timing and duration of the weather manipulations is indicated by black vertical bar.

Drought (D: 32 days without any precipitation) was simulated using rain-out shelters. Rain-out shelters were constructed with a steel frame and covered with transparent foil that permitted nearly 90% penetration of photosynthetically active radiation. Potential warming effects of shelters were lessened by building the roof at 80 cm height, allowing for wind through-flow thus near-surface air temperature was not significantly different below than outside of the shelters (pairwise t-test with Bonferroni correction: $p = 0.27$).

Heavy rain manipulation (R: total precipitation of 170 mm within a period of 14 days) was realized using a manually operated moveable irrigation system. Drop size and rainfall intensity resembled natural heavy rainfall events through application by Veejet 80100 nozzles, commonly used in erosion research (Kehl et al. 2005). The application of water was carried out twice every day to ensure constantly high soil water content (SWC) (~ 12.2 mm/d; half of it in the morning and the other half in the evening). If natural precipitation events occurred,

the amount of natural rain was subtracted from the respective daily dose of simulated rain thus, in sum, 170 mm rain in 14 days were not exceeded. Small plastic sheet pilings around each plot and around the manipulation blocks were installed to avoid leaching and lateral surface flow of water from the heavy rain treatment (simulated high SWC) to the drought treatment (simulated low SWC) or the ambient control (natural SWC).

SWC (as a proxy for effectiveness of weather treatments; Fig. 1) was measured at 5-10 cm depth with an ECH2O-5 sensor (Decagon, USA), which conducted hourly readings in five replications in the G4- plots. Due to technical problems, measurements of SWC started only five days after the start of the heavy rain treatment. Nevertheless we gained information about the most important time period at the end of the weather manipulations where greatest differences in soil moisture (due to treatments) were expected and observed.

Factor 2: functional diversity

Plant communities contain up to four species chosen from a five species pool: *Holcus lanatus* L., *Arrhenatherum elatius* (L.) P.B. ex J. et K. Presl, *Plantago lanceolata* L., *Lotus corniculatus*-group and *Geranium pratense* L. (Oberdorfer 2001). Species were chosen by their functional group (grass, forb, legume), their life-span (perennial), their overall importance in nearby and central European grassland systems, and their adaptation to the substrate. In April 2005, 2x2 m plots were planted with 100 individuals in a systematic hexagonal grid with 20 cm distance between neighbours. In autumn 2006, 1x1 m plots with 25 individuals were planted in the same pattern for monocultures of the two grass species. Original species combination was maintained by periodical weeding of non-planted species but spreading and succession of the planted species was freely allowed and resulted in an averaged cover per plot of nearly 75% on 26-Jun-2007 (Fig. 2). Mixtures containing *L. corniculatus* had an average cover of 95% whereas the other communities achieved nearly 70% cover (data not shown). The number of functional groups had a significant impact on total cover of the communities (one way ANOVA, $p < 0.001$), but no interaction effect between number of functional groups and weather manipulation was detectable ($p = 0.942$).

Four different diversity levels were realized in the experiment: monocultures of the grasses (G1-), two species mixtures (G2-) and four species mixtures without and with the legume species *L. corniculatus* (G4- and G4+, respectively). Community composition was:

G1-: 1x1 m plots; monocultures of *Holcus lanatus* (or *Arrhenatherum elatius*),

G2-: 2x2 m plots; two species (*Holcus lanatus* and *Arrhenatherum elatius*), one functional group (grass),

G4-: 2x2 m plots; four species (*Holcus lanatus*, *Arrhenatherum elatius*, *Plantago lanceolata*, *Geranium pratense*), two functional groups (grass, forb),

G4+: 2x2 m plots; four species (*Holcus lanatus*, *Arrhenatherum elatius*, *Plantago lanceolata*, *Lotus corniculatus*), three functional groups (grass, forb, legume).

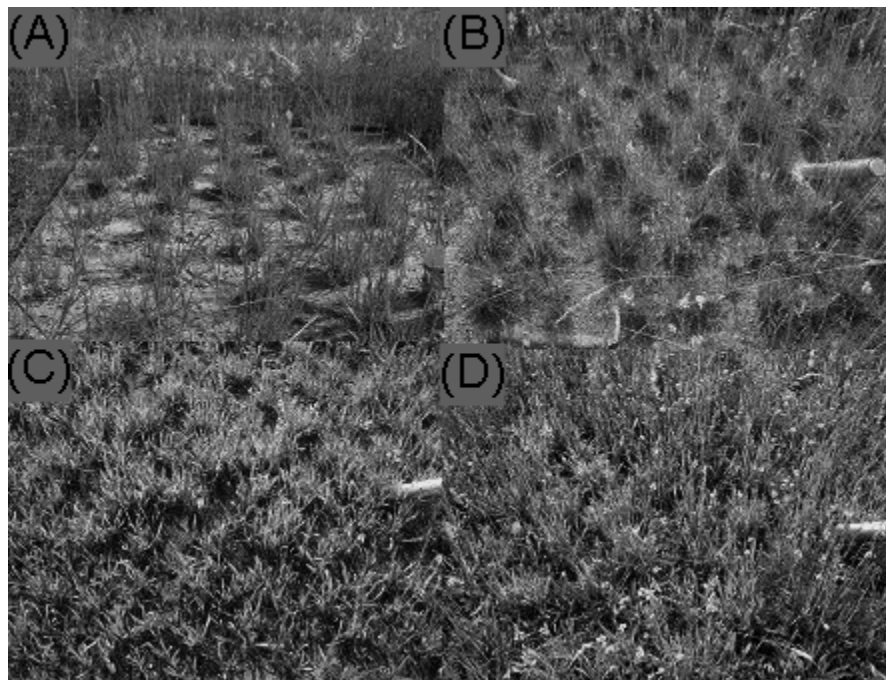


Figure 2 Photographs of four representative plots across the diversity gradient in the EVENT-Experiment (20-Jun-2007): *Holcus lanatus* in monoculture (G1-: **A**), a two species mixture with *H. lanatus* and *Arrhenatherum elatius* (G2-: **B**) and both four species mixtures with *H. lanatus*, *A. elatius*, *Plantago lanceolata* and *Geranium pratense* as a fourth species (G4-: **C**) or *Lotus corniculatus* as a fourth species (G4+: **D**).

Light reactions of photosynthesis using the chlorophyll a fluorescence method

The quantification of chlorophyll *a* fluorescence of photosystem II (PS II) is a non-invasive way of measuring the efficiency of light reactions *in situ* (Schreiber et al. 1986; Maxwell and Johnson 2000). Light intensity (PFD [$\mu\text{mol m}^{-2} \text{s}^{-1}$] = photon flux density; used here for light with a photosynthetic active wavelength range of $\lambda = 380\text{--}710 \text{ nm}$) changes within the canopy in reaction to diurnal cycles and canopy structure, thus leaves are exposed to varying light intensities.

In the field, we measured the fluorescence signal with two PAM-fluorometers (PAM = pulse-amplitude modulated photosynthesis yield analyzer; PAM-2100 and MINI-PAM by H. Walz

GmbH, Effeltrich, Germany) with leaf clip holders described by Bilger et al. (1995). We measured the fluorescence signal of *Holcus lanatus*, *Plantago lanceolata*, *Geranium pratense* and *Lotus corniculatus*. *Arrhenatherum elatius* was not measured because its leaf lamina was too narrow for PAM-measurements. Both fluorometers were calibrated against each other via light intensity and randomly chosen leaf samples prior to measurements. The light intensity was taken automatically as spot measurements by a microquantum sensor integrated in the leaf clip holders of both PAM devices. The fluorescence measurements per plot were conducted as follows: First we measured a fluorescence standard provided by H. Walz GmbH (for correction of absolute values) and then two leaves of five representative individuals per species (= 10 measurements per species and plot). We selected one individual in every corner of the inner square meter and one randomly chosen individual for measurements to obtain non-clustered results. We chose only fully developed leaves in the upper half of each plant and measured them between half-way across and the upper third of the leaf (in the direction of the leaf tip). During the measurements special care was taken not to change the ambient conditions, such as the angle of the leaf or its exposure to sun or shading. All measurements were conducted around solar noon (10:00 to 14:30). Measurements with two fluorometers were performed in opposite directions along the plots to ensure maximum randomization of measured parameters during the diurnal light course. We conducted fluorescence measurements on three days at the end of the treatment period (drought was applied for nearly one month and heavy rain for nearly two weeks) to reveal extreme weather effects on plant performance. We present data from the 20-Jun-2007 in the manuscript because this was the last day of weather manipulations and thus the one with the most extreme environmental impact. We provide data from a study in 2006 as Supplementary material.

As a direct response from the fluorometers, the effective quantum yield of light adapted leaves ($\Delta F/F_m'$, eq. 1), was obtained for every single leaf measured.

$$\frac{\Delta F}{F_m'} = \frac{(F_m' - F)}{F_m'} \quad (1)$$

Where F is fluorescence yield of the light-adapted leaf and F_m' is the maximum light-adapted fluorescence yield when a saturating light pulse ($\text{PFD} > 2000 \mu\text{mol m}^{-2} \text{s}^{-1}$, duration: 0.8 s) was superimposed on the prevailing ambient light levels (Genty et al. 1989; Schreiber and Bilger 1993). The effective quantum yield provides information on an individual measurement at ambient light conditions and (because values change with changing light intensity) is a non-comparable parameter under fluctuating, natural conditions. Potential quantum yield of

dark-adapted leaves (F_v/F_m), a comparable one-value-parameter, could not be measured experimentally in this study due to technical problems. Thus, we developed an alternative approach and extrapolated the effective quantum yield of light-adapted leaves to estimate the potential quantum yield; this parameter will be denoted as extrapolated potential quantum yield of PS II ($F_v/F_{m\text{expo}}$, eq. 2) in the following. For this, light response curves of $\Delta F/F_m'$ were fitted against PFD using a three-parametric, exponential decay regression.

$$\frac{\Delta F}{F_m'} = y_0 + a \cdot e^{(-b \cdot x)} \quad (2)$$

$F_v/F_{m\text{expo}}$ is calculated for $x = 0$, i.e. for extrapolating the fitted function for PFD = 0.

To gain the maximum electron transport rate (ETR_{max}), a further cardinal point of photosynthesis (Rascher et al. 2000; Peek et al. 2002; Rascher et al. 2004), we used a novel mathematical approach (eq. 3). It is based on the functions to calculate the apparent rate of electron transport from the effective quantum yield ($\text{ETR} = \Delta F/F_m' \cdot x \cdot 0.5 \cdot \text{absorption factor}$) and the regression equation to calculate ETR_{max} from ETR vs. PFD light response curves ($\text{ETR} = a \cdot (1 - e^{(-b \cdot x)})$) as described in Rascher et al. (2000).

$$\frac{\Delta F}{F_m'} = \frac{a \cdot (1 - e^{(-b \cdot x)})}{x \cdot 0.5 \cdot 0.77} \quad (3)$$

Here, x is the photosynthetically active radiation (PFD, $\lambda = 380\text{--}710$ nm). Under high-light conditions ($x \rightarrow \infty$), the exponential term $e^{(-b \cdot x)}$ becomes zero and the a -value directly reports the value for ETR_{max} . The mathematical calculation of ETR_{max} directly from light response curves of $\Delta F/F_m'$ considers the need for homoscedasticity of the data (which might be violated for light response curves of ETR vs. PFD). The factor 0.5 represents the assumption of equal excitation of both PS II and PS I and the factor 0.77 is the absorption factor, measured by an LI-1800-12 integrating sphere (LI-COR, Lincoln, NE, USA) on harvested leaves. The absorption factor reflected the averaged absorption of *H. lanatus* and *P. lanceolata* leaves over all three weather manipulations because there were no significant differences between treatments and leaves of different species. The factor was used for the calculations of ETR for all species measured. We used SigmaPlot 10.0 for the regression analyses and the calculation of cardinal points of photosynthesis.

Photosynthetic community response

We used the PAM-measurements of different species (*H. lanatus*, *P. lanceolata*, *G. pratense* and *L. corniculatus*) and calculated an averaged value from species-specific effective quantum yield measurements ($\Delta F/F_m'$) as a photosynthetic community response. We calculated the maximum electron transport rate of the whole community (community ETR_{max}) from community $\Delta F/F_m'$ using equation 3. This parameter (community ETR_{max}) might allow for the comparison between invasive harvest and non-invasive measurements at the community level.

*Aboveground net biomass production of *Holcus lanatus* individuals*

At the end of the weather treatment in July 2007, four individual plants (tussocks) of *H. lanatus* were harvested separately in every plot (to determine net biomass production of one individual = NP_{ind}), resulting in 20 individuals per weather manipulation and species richness level. They were dried for 72 hours at 70°C and weighed.

Statistical analysis

Data subsets

Due to the missing of monocultures in the heavy rain treatment, we used two subsets of data for the analyses of covariance (ANCOVA) of fluorescence data and for the analyses of variance (ANOVA) of individual biomass of *H. lanatus*. Subset 1 consisted of all weather treatments but excluded the diversity level monoculture (subset 1: G2-, G4-, G4+ in C, D and R). Subset 2 consisted only of the weather treatment drought and control but included all diversity levels (subset 2: G1-, G2-, G4-, G4+ in C and D).

Chlorophyll a fluorescence

We applied general linear models to our data and tested effects of different factors on photosynthetic response on species level (for *H. lanatus*) with different data sets. We used ANCOVA with light intensity (PPFD) as covariable and the effective quantum yield ($\Delta F/F_m'$) as dependent variable. First, we tested all available data for species-specific differences in $\Delta F/F_m'$ measurements (species as factor). Second, we tested effects of weather treatment and

diversity level (factors) on $\Delta F/F_m'$ measurements of the target species *H. lanatus* in subset 1 and 2.

We transformed $\Delta F/F_m'$ data exponentially to meet assumptions of normality and of homogeneity of variances required by ANCOVA. We used SPSS 11.5 for Windows (SPSS Inc., USA) for ANCOVA. The level of significance was set to $p = 0.05$.

We conducted pair-wise comparisons for significant differences in Chi-square-distribution (χ^2) with Wald-tests (see eq. 7 and Rascher et al. 2004) to specify differences (indicated by ANCOVA-results for $\Delta F/F_m'$) for the extrapolated potential quantum yield of PS II ($F_v/F_{m\text{expo}}$) and maximum electron transport rate (ETR_{max}) of *H. lanatus* and for community ETR_{max} under different abiotic and biotic conditions using Microsoft Office Excel 2003 (Microsoft Cooperation, USA).

$$W = \frac{(a_1 - a_2)}{\sqrt{[SE^2(a_1) + SE^2(a_2)]}} \quad (7)$$

The Wald-test compared two values with standard error: a_1 and a_2 (values for $F_v/F_{m\text{expo}}$ or ETR_{max}) are the parameters tested against each other and SE is the standard error of the mean of each parameter. Every factorial combination of weather treatment and diversity level was tested against each other to obtain information about statistical significant differences. The level of significance was set to $\alpha = 0.05$.

Individual biomass

For the statistical analysis of biomass of *H. lanatus* individuals (NP_{ind}) data were separated into subset 1 and 2. ANOVA combined with linear models were applied to test NP_{ind} for significant differences of weather treatment and diversity level (factors) while accounting for the fact that the diversity levels were nested within the weather treatment. The NP_{ind} data were tested for normality and heterogeneity of variances by examining normal qq-plots of the linear model as well as pp-qq-plots and residuals versus fitted plots. For subset 1 conditions of normality of data were not met, thus data were log transformed before analysis. The level of significance was set to $p = 0.05$.

To specify differences (indicated by ANOVA-results) in NP_{ind} of *H. lanatus*, we used Tukey's HSD post-hoc-comparisons. All statistical analyses related to NP_{ind} were performed using R 2.4.1 (R-Development-Core-Team 2006).

RESULTS

Species identity had a significant effect on the effective quantum yield ($\Delta F/F_m'$; ANCOVA over all data: $F_{3,1350} = 36.212$, $p < 0.001$) at the last day of the extreme weather manipulations (20-June-2007) as also species-specific fits in Figure 6 show. Thus all further results on species level report only about the target species *H. lanatus* which was available in nearly all diversity levels in three weather treatments and we used measurements on the other species only for the calculation of the community response.

Diversity level and weather treatment had significant effects on the effective quantum yield in leaves of *H. lanatus* (Fig. 3) along the gradient of light availability in both subsets of data and strong interactions between diversity and weather treatments occurred (Table 1 A, B).

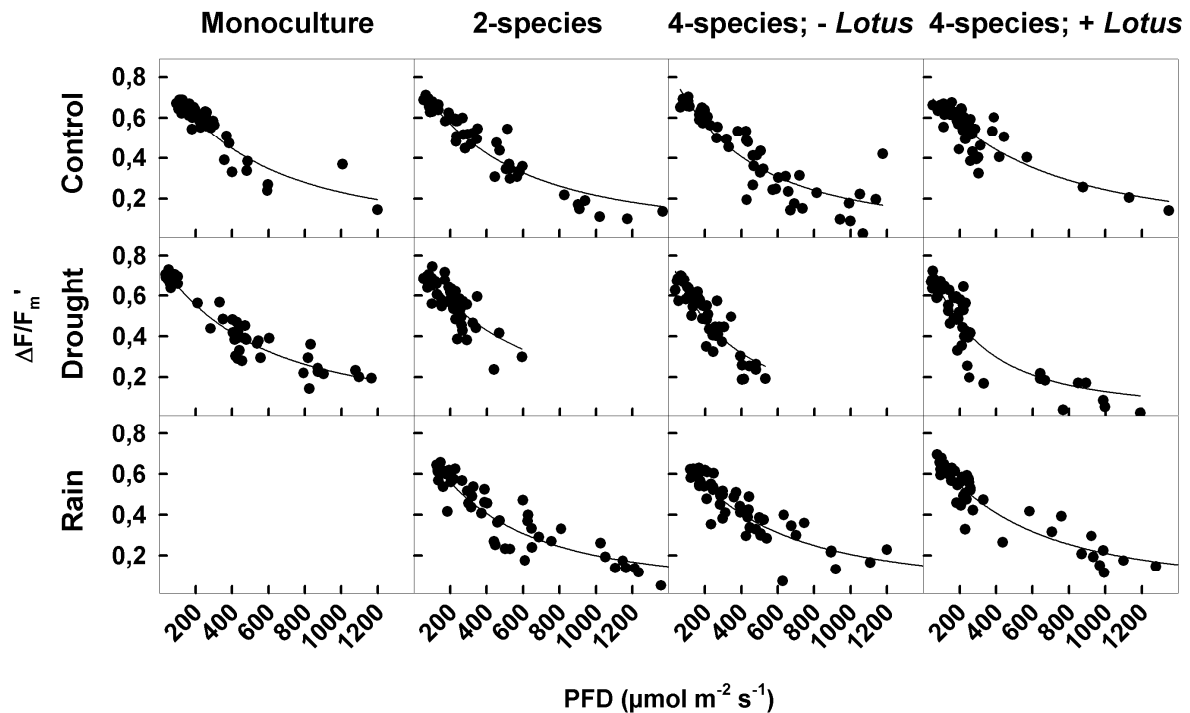


Figure 3 Effects of the different weather and diversity treatments on the effective quantum yield of photosystem II ($\Delta F/F_m'$ of PS II) of *Holcus lanatus* plotted against photon flux density (PFD) in the photosynthetic active range (wavelength: $\lambda = 380\text{--}710$ nm) on the last day of the manipulation period (20-Jun-2007). Each solid line indicates regressions fitted as described in equation 3. Weather treatments are shown row-wise, diversity levels are shown column-wise from monocultures (G1-) on the left side to the two-grass-species-communities (G2-) and the four species mixtures with two functional groups (G4-) up to the functionally most diverse communities with *Lotus corniculatus* included (G4+) on the right side of the panel.

Subsequent Wald-tests specified significant differences between diversity levels within a weather treatment for the two derived cardinal points of photosynthesis. Wald-tests revealed

differences in the maximum electron transport rate (ETR_{max}) (Table 2 A) but not in the extrapolated potential quantum yield of PS II ($F_v/F_{m\text{expo}}$) (Table 2 B).

Table 1 Effect of light intensity (PFD as covariable) and factors weather treatment (WT) and diversity level (DL) on effective quantum yield ($\Delta F/F_m'$) of *Holcus lanatus* tested with ANCOVA in two subsets of data: (A) subset 1 consisted of data for 2-species-mixtures (G2-), 4-species-mixtures without (G4-) and with (G4+) *Lotus corniculatus* grown in ambient control (C), drought (D) and heavy rain (R) treatment and (B) subset 2 consisted of data for all diversity levels (monoculture = G1-, G2-, G4-, G4+) grown in control and drought treatments.

(A) Subset 1		$\Delta F/F_m'$			(B) Subset 2		$\Delta F/F_m'$		
factor	d.f.	<i>MSQ</i>	<i>F</i>	<i>P</i>	d.f.	<i>MSQ</i>	<i>F</i>	<i>P</i>	
Weather treatment	1	20.850	1059.884	< 0.001	1	19.790	1181.199	< 0.001	
Diversity level	2	0.109	5.554	0.004	1	0.411	24.560	< 0.001	
WT x DL	2	0.112	5.685	0.004	3	0.187	11.146	< 0.001	
Residuals	450	0.020			399	0.017			

Under ambient conditions (C), diversity level had no significant effect on ETR_{max} . Lowest ETR_{max} values occurred under the impact of drought in both four species communities, significant differences are given below for the two data subsets.

Although ANCOVA showed significant effects of both experimental factors on the effective quantum yield ($\Delta F/F_m'$; Table 1), diversity and weather treatments had no clear effect on the extrapolated potential quantum yield of PS II ($F_v/F_{m\text{expo}}$; Table 2 B) if compared with Wald-tests. $F_v/F_{m\text{expo}}$ ranged from a minimum of 0.727 to a maximum of 0.836 and thus indicating good to maximum light quantum utilization (theoretically maximum value for potential quantum yield = 0.84). Although the standard errors were high at lower light intensity (< 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PFD; due to curve fitting, see eq. 2), we tested the two most extreme $F_v/F_{m\text{expo}}$

Table 2 (A) Maximum electron transport rate (ETR_{max}) and (B) extrapolated potential quantum yield of PS II ($F_v/F_{m\text{expo}}$) in leaves of *Holcus lanatus* from different diversity levels (columns) and weather treatments (rows) at the end of the treatment period. Both cardinal points of photosynthesis were calculated from $\Delta F/F_m'$ (see eq. 2, 3) and are given with standard error of the mean (SE), n = 50.

(A)	G1-	S.E.	G2-	S.E.	G4-	S.E.	G4+	S.E.
C	91.51	6.32	85.34	4.48	76.75	5.13	98.94	10.72
D	86.61	3.92	89.33	8.57	54.54	3.58	49.84	4.38
R	n.a.		78.26	5.41	81.11	5.77	83.76	6.41

(B)	G1-	S.E.	G2-	S.E.	G4-	S.E.	G4+	S.E.
C	0.822	0.102	0.757	0.153	0.813	0.103	0.727	0.182
D	0.759	0.069	0.748	15.083	0.745	2.119	0.785	0.078
R	n.a.		0.751	0.105	0.758	0.070	0.751	0.055

Weather treatments: C = ambient control, R = heavy rain, D = drought,

Diversity levels: G1- = *Holcus lanatus* monoculture, G2- = *H. lanatus* + *Arrhenatherum elatius*, G4- = *H. lanatus*, *A. elatius*, *Plantago lanceolata* and *Geranium pratense*, G4+ = *H. lanatus*, *A. elatius*, *P. lanceolata* and *Lotus corniculatus*, n.a. = not available

values against each other using the Wald-test to check if significant differences between treatments may have occurred in case of low standard errors, but found no significant effect.

Diversity level and weather treatment both had significant effects on aboveground net biomass production of *H. lanatus* individuals (NP_{ind}) (Table 3 A, B; Fig. 4, 5). Lowest NP_{ind} was observed in plots exposed to heavy rain. Highest NP_{ind} was achieved in the two species mixture (G2-) irrespective of weather manipulation.

Table 3 Effect of weather treatment and diversity level on individual biomass production of *Holcus lanatus* plants (NP_{ind}) tested with ANOVA in two subsets of data: **(A)** first subset consisting of data for 2-species-mixtures (G2-), 4-species-mixtures without (G4-) and with (G4+) *Lotus corniculatus* grown in ambient control (C), drought (D) and heavy rain (R) treatment and **(B)** the second subset consisting of data for all diversity levels (monoculture = G1- and G2-, G4-, G4+) grown in control and drought treatment.

(A) Subset 1					(B) Subset 2				
NP_{ind}					NP_{ind}				
factor	d.f.	<i>MSQ</i>	<i>F</i>	<i>p</i>	d.f.	<i>MSQ</i>	<i>F</i>	<i>p</i>	
Weather treatment	2	5.93	8.072	<0.001	1	34.34	9.570	0.002	
Diversity level	2	8.02	10.917	<0.001	3	33.82	9.424	<0.001	
WT x DL	4	2.71	3.684	0.007	3	10.22	2.849	0.040	

Link between photosynthetic energy conversion and biomass production for H. lanatus

Considering subset 1 to investigate effects of all weather treatments in three out of four diversity levels (G2-, G4- and G4+, without monocultures), we found a severe reduction in ETR_{max} for *H. lanatus* in both four species communities compared to G2- communities under drought stress. The difference was significant between G2- and G4+ ($\chi^2 = 4.10$, $\alpha \leq 0.05$) but not between G2- and G4- ($\chi^2 = 3.75$, $\alpha = 0.1$) (Table 2 A). Comparing *within* a diversity level under environmental stress, we found that ETR_{max} was severely reduced in both four species communities under drought compared to the same species composition under ambient conditions - also a significant reduction occurred only in the four species community with a legume species (G4+: $\chi^2 = 4.24$ $\alpha = 0.05$ but G4-: $\chi^2 = 3.55$ $\alpha = 0.1$). Contrary to the ETR_{max} response to drought, an excess of water due to the heavy rain treatment had no such effect on ETR_{max} ($\chi^2 = -0.56$ to 1.22 , $\alpha > 0.05$) either within the rain treatment (G2- vs. G4- vs. G4+) or within a given diversity level (C vs. R in G2-, G4-, G4+).

In contrast to ETR_{max} , heavy rain decreased averaged productivity of *H. lanatus* individuals significantly (NP_{ind} , Fig. 4) whereas drought stress did not (averaged values *within* weather treatments: C vs. R, D vs. R: both $p \leq 0.016$). Significantly higher averaged NP_{ind} occurred at the lowest species richness level of subset 1 (averaged values *within* diversity levels: G4- vs. G2-, G4+ vs. G2-: both $p < 0.001$). In the four species communities, NP_{ind} of *H. lanatus* was

not affected by community composition: no significant differences occurred between the presence (G4+) or absence (G4-) of the legume species *L. corniculatus* (G4- vs. G4+: $p = 0.978$).

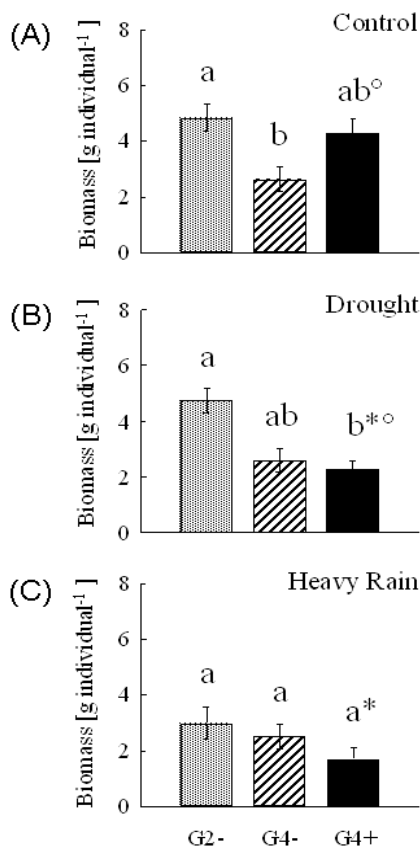


Figure 4 Subset 1: Net biomass production of *Holcus lanatus* individuals (NP_{ind} [g]) grown in 2-species mixtures (G2-), 4-species-mixtures without (G4-) and 4-species-mixtures with (G4+) the legume *Lotus corniculatus* under the weather treatments (A) ambient control, (B) drought and (C) heavy rain. Data are mean \pm 1 standard error of the mean ($n = 5$), different letters indicate horizontal differences (*within* a weather treatment), different symbols indicate vertical differences (*within* a diversity level) from post-hoc tests ($p < 0.05$).

Diversity levels affected biomass accumulation *within* the weather treatments significantly (Fig. 4). Under ambient conditions individuals grown in G2- had significantly higher biomass than those grown in the four species communities without a legume species (G4-). This response was not found under drought. Under drought stress, individuals grown in G2- had no significantly higher NP_{ind} than individuals in G4- ($p = 0.09$) but significantly higher NP_{ind} than individuals grown in G4+. Within the heavy rain treatment, NP_{ind} was always very low and the diversity level had no effect on the individual biomass.

Within the drought treatment, the integrated (biomass) and the non-invasive instant (fluorescence) method gave the same direction and significance of results. Within the diversity levels, negative effects of drought on *H. lanatus* compared to the same species under ambient

conditions were detected with both methods, but whereas the non-invasive method showed significant differences, the invasive measurements only indicated trends. Heavy rain reduced NP_{ind} significantly in more diverse communities whereas it had no effect on ETR_{max} of *H. lanatus*.

Buffering effect of surrounding species richness on H. lanatus under drought stress

A comparison for ETR_{max} and NP_{ind} of *H. lanatus* across the whole diversity gradient of the experiment was possible for subset 2, comparing all diversity levels in drought treatment and control, but not between heavy rain and control.

ETR_{max} values under ambient conditions (Table 2A) remained relatively stable and without any significant differences related to diversity levels ($\alpha > 0.05$) if monocultures were included in the analysis. No difference in ETR_{max} was observed between the monocultures (G1-) and two species mixtures (G2-) under ambient or drought stressed conditions. Under drought stress both four species communities (G4- and G4+) showed significantly reduced ETR_{max} compared to monocultures ($\chi^2 > 6.00$, $\alpha = 0.05$).

Comparing drought and control (subset 2, Fig. 5), averaged individual biomass production decreased significantly under extreme drought. *H. lanatus* showed lower average NP_{ind} when grown together with the *A. elatius*, *P. lanceolata* and *G. pratense* (G4-) in comparison to individuals which grew in monoculture (G1-) or together with *A. elatius* (G2-). NP_{ind} was about 1.5 g per individual lower in the four species mixtures with *L. corniculatus* (G4+) compared to the two species mixtures (G2-). Under drought stress, the species richness level significantly modified the biomass response. While under ambient conditions, individuals in monocultures had significantly higher biomass than those in the four species mixtures without a legume species (G4-) the effect vanished under drought stress. Furthermore, the significant difference in NP_{ind} between individuals in G2- and individuals in G4+ only occurred under drought.

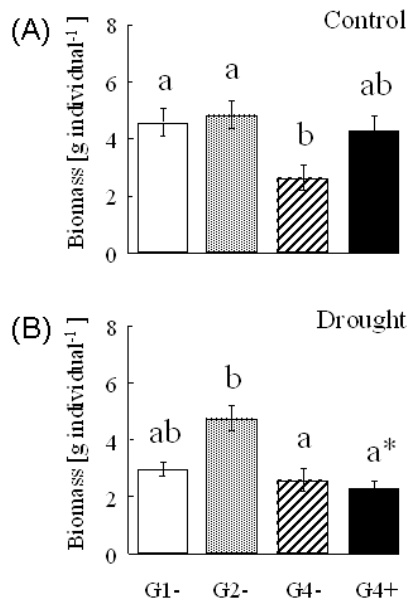


Figure 5 Subset 2: Net biomass production of *Holcus lanatus* individuals (NP_{ind} [g]) grown in monocultures (G1-), 2-species mixtures (G2-), 4-species-mixtures without (G4-) and 4-species-mixtures with (G4+) the legume *Lotus corniculatus* under the weather treatments (A) ambient control and (B) drought. Data are mean \pm 1 standard error of the mean (n = 5), different letters indicate horizontal differences (*within* a weather treatment), different symbols indicate vertical differences (*within* a diversity level) from post-hoc tests ($p < 0.05$).

No buffering effect of increasing species richness on the performance of *H. lanatus* individuals occurred under drought stress. Drought stress resulted in a severe decrease in ETR_{max} accompanied by a decrease of NP_{ind} which was obvious in both four species communities whereas monocultures or pure grass mixtures did not show such a parallel decrease in both parameters.

Photosynthetic community response to extreme weather events

Community ETR_{max} (which was derived from community $\Delta F/F_m'$, Fig. 6, averaged from species-specific $\Delta F/F_m'$ in leaves of *H. lanatus*, *P. lanceolata*, *G. pratense* and *L. corniculatus* by using eq. 3) was relatively stable and showed only little response to the extreme weather treatments (Table 4).

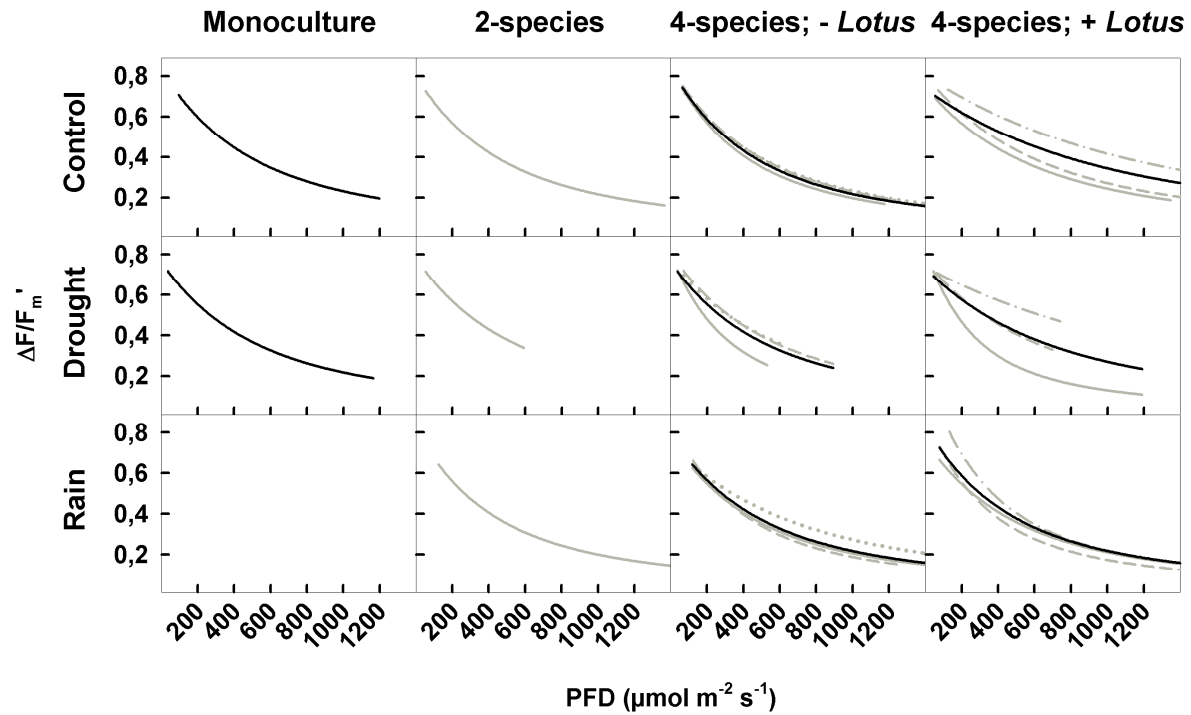


Figure 6 Effects of the different weather and diversity treatments on the effective quantum yield of communities (community $\Delta F/F_m'$) plotted against photon flux density (PFD) in the photosynthetic active range (wavelength: $\lambda = 380\text{--}710$ nm) on the last day of the manipulation period (20-Jun-2007). Weather treatments are shown row-wise, diversity levels are shown column-wise from monocultures of *Holcus lanatus* (G1-) on the left side to the 2-species-communities (G2-; only *H. lanatus* was measured) and the four species mixtures with two functional groups (G4-) up to the functionally most diverse communities with *Lotus corniculatus* included (G4+) on the right side of the panel. Community ETR_{\max} (Table 4) was derived from the regression curves mathematically using eq. 3. Black solid lines indicate community response whereas grey colour indicates a species-specific response within a mixture: grey-solid = *H. lanatus*, grey-dashed = *Plantago lanceolata*, grey-dotted = *Geranium pratense* and grey-dot-dashed = *L. corniculatus*.

Pair-wise comparisons showed no significant differences except for community ETR_{\max} in the four species communities with a legume species (G4+). Community ETR_{\max} in G4+ in the control was significantly higher than in the other communities under ambient conditions and than community ETR_{\max} of G4+ in the heavy rain treatment ($\chi^2 > 5.06$, $\alpha \leq 0.05$). Community ETR_{\max} of drought treated G4+ was not significantly different to G4+ in the control ($\chi^2 = 2.57$, $\alpha = 0.2$). Negative effects of drought on ETR_{\max} of *H. lanatus* were ameliorated by neighbouring species thus overall community performance was not harmed.

Table 4 Maximum electron transport rates of communities (community ETR_{\max}) calculated from community $\Delta F/F_m'$ (see eq. 3) with 1 standard error of the mean in different diversity levels (columns) and weather treatments (rows) at the end of the treatment period.

	G1-	S.E.	G2-	S.E.	G4-	S.E.	G4+	S.E.
C	91.51	6.32	85.34	4.48	85.40	3.76	158.95	12.82
D	86.61	3.92	89.33	8.57	86.48	5.96	113.15	12.34
R	n.a.		78.26	5.41	86.18	4.66	85.96	5.46

DISCUSSION

*Effect of drought stress on photosynthetic efficiency and biomass production of *H. lanatus**

Our **first hypothesis** asked whether we find reductions in photosynthetic performance under extreme weather stress in the target species *H. lanatus* when measuring the chlorophyll *a* fluorescence signal and consequently whether reduced photosynthetic efficiency (instant measure) results in reduced biomass per individual (integrative measure). Drought can have negative effects on leaf area and, as a feedback, can reduce CO₂ assimilation and thus further biomass production (Hsiao 1973). CO₂ assimilation is correlated to chlorophyll *a* fluorescence under lab conditions and although the relationship is weaker under field conditions, measurements of fluorescence are often indicative for the rate of photosynthesis (Maxwell and Johnson 2000). Thus, we consider this an important question to address as fluorescence can be measured quickly and non-invasively (whereas the biomass harvest is highly invasive and laborious). Fluorescence measurements could provide an early screening indication of potential biomass reduction if the two parameters were found to be clearly correlated in the field.

We found a weak relationship between ETR_{max} and NP_{ind} under drought stress and no link at all between these parameters in the heavy rain treatment. Despite the theoretical link (Hsiao 1973; Maxwell and Johnson 2000), decreases in photosynthetic efficiency and biomass production seemed to be driven by different constraints, such that fluorescence would not form a good non-invasive surrogate for measuring productivity invasively. We detected signs of drought stress in *H. lanatus* under field conditions in most diverse communities (G4-, G4+) when measuring ETR_{max} but not for $F_v/F_{m\text{exp}}$ (Table 2 A, B). Negative effects of drought on electron transport rates were confirmed by reduced individual biomass production (NP_{ind}, Fig. 5), although significantly only in four species communities with a legume species (G4+). The negative effect of severe drought on the photosynthetic performance (ETR_{max}) of *H. lanatus* in four species communities was consistent over two consecutive years (in 2006: Kreyling et al., Table S1; Fig. S1 in Supplementary material) and thus does not represent a single-year exception. Interestingly, it was not possible to detect drought stress via the fluorescence signal in species poor communities (G1-, G2-), containing only one functional group (grasses), although there was a trend to lower NP_{ind} in monocultures and two species mixtures, too (Fig. 5).

A possible explanation for reduced ETR_{max} could be an earlier senescence of *H. lanatus*. Senescent or older leaves usually have a lower photochemical efficiency (Figuerola et al.

1997; Waldhoff et al. 2002). Drought is known to induce early senescence in a wide variety of plant species, e.g. in *Salvia officinalis* L. (sage, forb) (Munne-Bosch et al. 2001), *Triticum aestivum* L. (wheat, grass) (Shah and Paulsen 2003) or *Cicer arietinum* L. (chickpea, legume) (Macar and Ekmekci 2008). Maize plants have been found to respond to an artificial drought (root detachment) with a significant reduction in ETR_{max} that is stronger in younger than in older leaves (Xu et al. 2008), indicating a stronger reaction to drought of not naturally senescent leaves. A study in the EVENT-Experiment on the response of flowering phenology to extreme weather events revealed earlier flowering and a reduction in flowering length in *H. lanatus* under drought stress (although no effect of diversity levels) (Jentsch et al. 2009). This phenological shift suggests that *H. lanatus* may have experienced early senescence in the EVENT-Experiment under drought stress. Data on photosynthetic pigment content, on the other hand, did not confirm an earlier senescence (personal communication Julia Walter).

Effect of heavy rain events on photosynthetic efficiency and biomass production of H. lanatus

Negative effects of heavy rain treatment on *H. lanatus* were not found for chlorophyll *a* fluorescence (Table 1, 2) albeit strong reductions in NP_{ind} (Fig. 4). Pociecha et al. (2008) found that an excess of soil water constrained both growth as well as the photosynthetic apparatus in field bean (*Vicia faba* L. *minor*, legume), but that the strength of impact depended strongly on leaf age. The heavy rain treatment in the EVENT-Experiment increased the soil water content and waterlogged the soil for more than one week (Fig. 1). Thus, we expected a reduction in the photosynthetic capacity but it was not observed in our study whereas we found a reduction in individual biomass of *H. lanatus* (NP_{ind}) in the heavy rain treatment, in agreement with Pociecha et al. (2008). They related negative effects of flooding on biomass production to a disturbed hormonal equilibrium and restrictions of metabolic processes due to root anoxia. Shi et al. (2008) stated that it is mainly a lack of oxygen what leads to biomass reductions under root limited conditions in *Lycopersion esculentum* (tomato, forb). Thus, short-term root anoxia may have hampered NP_{ind} in *H. lanatus* but was possibly not severe enough to negatively affect the photosynthetic light use efficiency.

Buffering effect of surrounding species richness on H. lanatus under drought stress

Contrary to our expectations, *H. lanatus* performed worse under drought stress combined with higher surrounding species richness both in terms of reduced ETR_{max} (Table 2 A) and NP_{ind} (Fig. 4, 5). Thus, we cannot confirm our **second hypothesis**, which predicted a better

performance of *H. lanatus* in more diverse communities under severe drought stress. For temperate grass species, the process of facilitation is a function of a species having both a low tolerance to a particular abiotic stress and a strong competitive-response ability (Liancourt et al. 2005). *H. lanatus* was expected to fulfill these conditions, because it is severely affected by drought and a strong competitor in benign environments (Beddows 1961). However, we found no sign that *H. lanatus* profited from facilitative effects linked to higher species richness or compositional changes.

The negative effect of increasing diversity on ETR_{max} and the reductions in NP_{ind} in drought stressed *H. lanatus* may be related to an increase in belowground competition in species-rich communities. Aboveground biomass per plot (represented by a significantly higher cover per plot in G4+ communities; data not shown) did not seem to determine all of the stress response to drought, as a negative effect was found in both four species communities (not just in the four species communities with a legume species). Several studies have shown higher complementarity between species in more diverse communities and that this can lead to more efficient total resource use as published by Berendse (1979), Loreau & Hector (2001) and by De Boeck *et al.* (2006) for higher water use efficiency (WUE). Verheyen et al. (2008) found in the Swedish BIODDEPTH biodiversity field experiment that more diverse communities showed a higher WUE under mild drought stress, but that they were more swiftly negatively affected when the drought stress increased. Complementarity under environmental stress is suggested to be most important, if species richness drops below a critical level of ten species for grasslands (Schwartz et al. 2000; Kahmen et al. 2005; De Boeck et al. 2007). Thus, the four-species-systems fall in the range of maximum resource complementarity at the community level (hence stronger belowground activity) and this might in turn affect single species performance negatively. Indeed data on belowground processes in grassland and heath communities in the EVENT-Experiment (Kreyling et al. 2008b) showed an increase in root accumulation and enzyme activity with increasing functional diversity irrespective of weather treatment, which indicates a better niche occupancy in the more diverse communities.

Furthermore, *H. lanatus* is known to be drought sensitive and is an indicator of medium to high soil moisture with an Ellenberg F-value = 6, whereas the neighbouring species in the four species communities have lower Ellenberg F-values of 5, x = indifferent, 5 and 4 for *Arrhenatherum elatius*, *Plantago lanceolata*, *Geranium pratense* and *Lotus corniculatus*, respectively (FloraWeb, BfN 2008) (Ellenberg indicator values; ranging from 1 (= avoiding)

to 9 (= loving) for different environmental parameters as for soil moisture (F, from German “Feuchte”). Overall our data suggest that the competitive strength of *H. lanatus* was reduced in the more diverse communities (G4- and G4+) where it suffered more than the other species from extreme drought (Fig. 6).

Community response to extreme weather events

As Balvanera et al. (2006) emphasise, biodiversity effects often depend on the level of abstraction focussed on: individual and population effects are often very different from effects at community level. According to the insurance hypothesis (Naeem and Li 1997; Yachi and Loreau 1999) - which predicts higher stability and resilience of more diverse communities in the face of disturbance - negative effects on one species can be ameliorated by an increased performance of another species in species (or genotypic) rich communities. This effect was indeed observed for the photosynthetic response of the community in our experiment: only the performance of *H. lanatus* was reduced under drought stress while the neighbouring species did not perform worse (Fig. 6). Thus, on community level, no negative effects of extreme weather events were observed (community ETR_{max} , Table 4), confirming our **third hypothesis**. Alike, the overall productivity in the plots was not reduced due to the weather manipulations in 2007 (Kreyling et al. 2008a) which indicates that other species profited from the photosynthetic limitations of *H. lanatus* under drought stress. Our results confirming those from a greenhouse study where the community response (averaged potential quantum yield of PS II, F_v/F_m) was also not affected by species richness or drought stress under ambient temperatures. The community's effective quantum yield ($\Delta F/F_m' \triangleq \Phi_{PSII}$) decreased in the course of the drought period (24 d), although at the end no significant differences between monocultures and communities with ~4.5 species have been detected (Zavalloni et al. 2009). Results from that study and our own indicate that the measurement of chlorophyll *a* fluorescence provide a useful tool to evaluate community resistance to drought stress under controlled and semi-natural conditions.

In conclusion we can state that the non-invasive measurement of chlorophyll *a* fluorescence under semi-natural field conditions is a helpful tool to assess reductions in performance of plant populations related to drought stress but not for reactions to waterlogged soil conditions. Thus, a correlation of effects detected by the instant (non-invasive chlorophyll *a* fluorescence) and the integrated (invasive biomass harvest) method was not possible for both environmental

stresses applied. Under severe drought stress, the maximum electron transport rate (ETR_{max}) reflected the worse performance of *H. lanatus* in the more diverse communities and low NP_{ind} confirmed this finding. We found no evidence for positive biodiversity effects on the performance of the target species *H. lanatus*, either under ambient or extreme weather conditions. Increasing diversity affected integrated and instant measures of *H. lanatus* negatively whereas no such effect was found for the instant community response. Functional diversity, acting as a buffer against disturbance on the community level, is important for insuring ecosystem stability at the community level (sensu insurance hypothesis, McNaughton 1977; Naeem and Li 1997; Yachi and Loreau 1999) but it had negative effects on the population of the grass species we investigated. The sensitivity of *H. lanatus* to extreme drought (as a result of climate change) may have substantial ecological effects on the distribution of this wide spread species in grassland communities in Germany and Europe and could affect grassland systems in a non-predictable way where this species is relatively abundant.

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SUPPLEMENTARY MATERIAL

Table S1 Parameters of the instant light response curves of *Holcus lanatus*, measured at the end of the weather manipulation period in 2006, separated by diversity level for each weather manipulations. Note that no significant difference between any diversity level was found within any weather manipulation. Michaelis-Menten equations ($ETR = a * PAR / (b + PAR)$) were fitted using quantile regression. Significance obtained by a pair-wise permutation procedure with 1000 permutations (data kindly provided by Jürgen Kreyling).

parameter		Drought	Roof Control	p	Heavy Rainfall	Control	p
a	G2 ⁻	160.4	219.5	0.024	109.9	135.5	0.097
	G4 ⁻	141.6	206.2	0.007	136.4	119.5	0.125
	G4 ⁺	131.8	NA		97.5	91.8	0.220
b	G2 ⁻	524.8	747.9	0.025	336.5	410.1	0.176
	G4 ⁻	434.3	694.3	0.007	427.7	351.4	0.092
	G4 ⁺	428.5	NA		290	274.3	0.287

G2⁻ = two species communities, G4⁻ = four species communities without a legume species, G4⁺ = four species communities with a legume species

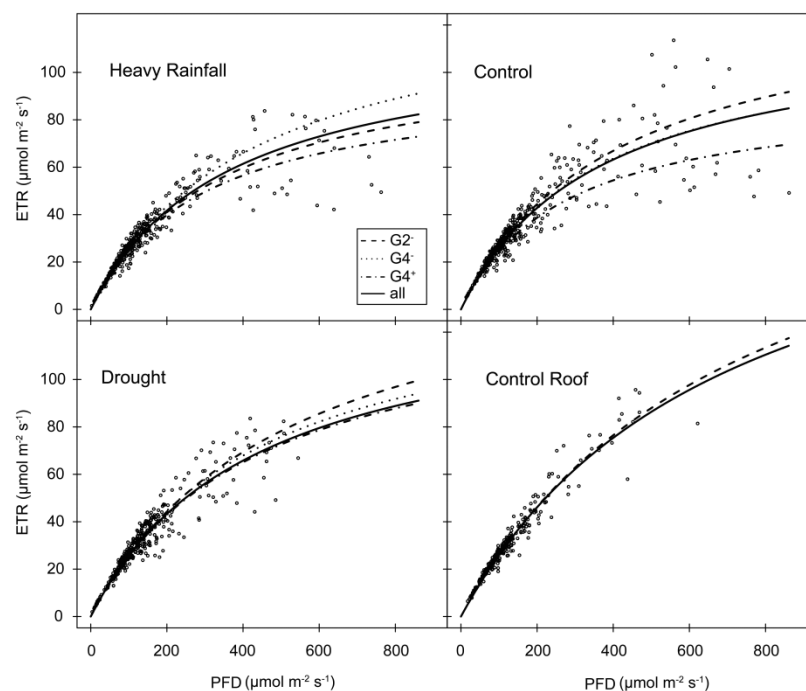


Figure S1 Apparent electron transport rate (ETR) in leaves of *Holcus lanatus* in different weather treatments and diversity levels in 2006. The study was conducted by Kreyling et al. in 2006 in the EVENT-Experiment. Drought resulted in significant reductions of ETR_{max} (parameters *a*) and ascending slope of light response characteristics (parameter *b*) described by the light response curves of *Holcus lanatus* compared to roof artefact control over all functional diversity levels (see Table S3).

Light response characteristics of *Holcus lanatus*. Michaelis-Menten equations are fitted by quantile regression on the electron transport rate (ETR in $\mu\text{mol m}^{-2} \text{s}^{-1}$) against photosynthetically active radiation (PAR in $\mu\text{mol m}^{-2} \text{s}^{-1}$): $ETR = a * PAR / (b + PAR)$. Solid curves: fits over all communities, dashed curves: communities containing two species of one growth form (G2⁻), dotted curves: communities containing four species of two growth forms (G4⁻), dot-dashed: communities containing four species of two growth forms, one legume (G4⁺).

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MANUSCRIPT 2

PRESENCE OF A LEGUME SPECIES REDUCES THE ECOPHYSIOLOGICAL
PERFORMANCE OF *HOLCUS LANATUS* DURING A DROUGHT, BUT
SPEEDS UP RECOVERY AFTER DROUGHT STRESS

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ABSTRACT

Current climate change increases the likelihood of extreme weather events and consequently the abiotic stress exerted on plants in many regions of the world. An increase in stress levels can induce shifts in plant-plant interactions resulting in more facilitation and less competition. Studying facilitation in communities with different diversity levels under extreme conditions is an emerging topic in ecology. We investigated productivity and the ecophysiological performance of the grass *Holcus lanatus* growing in communities with different diversity levels through a period of extreme drought and in the recovery phase after drought. We measured leaf water potential, chlorophyll *a* fluorescence, leaf pigment content, total protein and nitrogen concentrations, $\delta^{15}\text{N}$, the productivity of *H. lanatus* individuals and community productivity in all diversity levels. Drought treatment significantly reduced the water availability for *H. lanatus* in every plant community, with the greatest reduction occurring in communities that included a legume species. Communities with a legume species were most productive, irrespective of weather manipulation. Protein content, N-concentrations and $\delta^{15}\text{N}$ values in leaves of *H. lanatus* under ambient conditions indicated a facilitative effect of the legume species on N-supply of the grass species. This facilitative effect did not show up in drought stressed legume communities. *H. lanatus* that grew in communities with a legume species showed the significantly lowest maximum quantum efficiency (F_v/F_m) during the drought period, but, by contrast, also showed a quicker recovery of F_v/F_m after the drought period compared to individuals that were growing in monocultures. *H. lanatus* growing in monocultures and two-species communities produced more biomass per individual than in both four-species communities. The biomass production in four-species communities with a legume species was higher than in four-species communities without a legume. The findings suggest that the presence of a legume species reduces the performance of neighbouring species under extreme drought conditions. High productivity in these communities might enhance inter-specific competition due to increasing resource limitation. In the recovery phase after drought, presence of the legume species speeds up recovery, an effect that might be related to higher nitrogen availability in these communities. The increase in facilitation in the recovery phase could be one reason for improved resilience and recovery in functionally more diverse communities.

Keywords

Chlorophyll *a* fluorescence, recovery, climate change, EVENT-experiments, extreme weather event, *Lotus corniculatus*

Abbreviation list

C	= ambient control
D	= drought treatment
Doy	= day of the year
F_m	= maximum fluorescence yield of the dark adapted leaf
F_m'	= maximum fluorescence yield of the light adapted leaf
F_0	= steady state fluorescence yield of the dark adapted leaf
F_t	= steady state fluorescence yield of the light adapted leaf
F_v	= variable fluorescence yield of the dark adapted leaf
F_v/F_m	= potential maximum quantum yield of photosystem II
LWP [MPa]	= leaf water potential
NP_{ind}	= aboveground net biomass production of individuals [g/tussock]
PPFD [$\mu\text{mol m}^{-2}\text{s}^{-1}$]	= photosynthetically active photon flux density
W	= weekly average precipitation

INTRODUCTION

Extreme weather events such as drought are likely to increase in frequency and magnitude in the near future in many parts of the world due to global climate change (Trenberth et al., 2003; Schär et al., 2004). Drought is one of the major limitations for plant growth, affecting vegetation structure, plant productivity and interactions between plants (Chaves et al., 2004). Plants can interact via competition (negative) or facilitation (positive) and it has been observed, that facilitation becomes the dominating mode of plant interaction under adverse conditions, while competition is the more important driver of community organization under more favourable conditions. The balance between competition and facilitation along an environmental gradient is summarized as the stress gradient hypothesis (SGH, Bertness and Callaway, 1994). The hypothesis has been supported by different studies (Eckstein, 2005; Kikvidze et al., 2006; Sthultz et al., 2007; Eranen et al., 2008; le Roux et al., 2008). However, recent studies have shown that the outcome of interactions is highly context-dependent (site condition, species, type of stressor) thereby questioning the generality of the stress gradient hypothesis (Armas et al., 2005; Weedon et al., 2008; Maestre et al., 2009). In many cases, an increase in stress by a reduction of the limiting factor, e.g. water in arid environments, has even been found to lead to increased competition instead of facilitation (Tielbörger et al., 2000; Ludwig et al., 2004; Maestre et al. 2004).

The study of plant-plant interactions is linked to “some of the most important current ecological issues, including the relationship between biodiversity and ecosystem function, and the impacts of global change” (Brooker et al., 2008). However, most studies focus on pair-wise plant-plant interactions (Fotelli et al., 2001; Britton et al., 2003; Fernandez et al., 2007; Cavieres et al., 2008) and only a few studies investigate the effect of a varying number of neighbouring species or functional groups on the performance of an individual target species. Ecological theories such as the insurance hypothesis (Yachi and Loreau, 1999) state that higher diversity increases the probability of maintaining the functioning and stability of an ecosystem in a fluctuating environment. This is partly explained by a higher trait asynchrony and redundancy and partly by increased positive interactions in more diverse communities (Tilman and Downing, 1994; Yachi and Loreau, 1999). The positive relationship between ecosystem functioning and biodiversity is related to (i) niche complementarity (higher performance through facilitation or resource partitioning) and (ii) the selection effect (higher probability of including a good performer in more diverse communities) both of which are explained comprehensively by Loreau et al. (2001). Legumes are often key species for

enhanced stability in plant communities, as they act as facilitators (enhance productivity and N-nutrition of neighbouring species) in plant communities under non-stress conditions (Spehn et al., 2002; Hector et al., 2007; Temperton et al., 2007; Dybzinski et al., 2008). However, their role on the performance of neighbours under abiotic stress conditions has rarely been studied.

Cardinale et al. (2002) stated that “facilitation may be a key mechanism by which biodiversity enhances the performance of ecosystems” thus linking facilitation research to community ecology. Maestre et al. (2009) and Brooker et al. (2008) highlighted the need for extending research on plant interactions from the individual level up to the community level. In plant communities with higher diversity levels, non-linear effects and the indirect effects of facilitation also become apparent (Michalet, 2006). For instance, Mulder et al. (2001) found an increase in positive interactions in more diverse bryophyte communities during drought. Furthermore, both species- and genetic-diversity speed up the recovery of plant communities and therefore enhance resilience as has been found by the few existing studies investigating post-stress community recovery (Tilman et al., 1996; Reusch et al., 2005).

This study addresses effects of neighbouring species richness and composition on productivity and on the ecophysiological performance of *Holcus lanatus* (Yorkshire fog, Poaceae) under extreme drought and in the post-drought recovery phase. *H. lanatus* is a widely distributed perennial grass with a wide ecological range, mainly occurring in moist habitats on relatively fertile and moderate acidic soils (pH 5-6) (Grime et al., 1988). Our aim was to link facilitation and biodiversity research by measuring the response of one species within differently composed communities varying in species and functional trait number. We measured physiological parameters (chlorophyll *a* fluorescence, leaf water potential, content of photosynthetic pigments) as well as biomass production per individual to quantify sensitivity to stress and the course of recovery. To investigate community response and a possible facilitative effect of legume presence, we assessed community productivity and nitrogen data. Our study was based on three hypotheses:

- (i) Community diversity buffers the negative effects of drought stress on *H. lanatus* due to an enhancement of facilitative effects, such as shading or hydraulic lift.
- (ii) The presence of a legume species further enhances the buffering capacity of community diversity, as legumes are known to act facilitative under non-stress conditions.

- (iii) The recovery of *H. lanatus* after drought is speeded up in more diverse communities, due to a better overall performance and improved resilience of more diverse communities.

MATERIAL AND METHODS

Experimental design

Our study was conducted as part of the EVENT-experiments, which investigate the effects of simulated extreme weather events and species diversity on ecosystem functions (Jentsch et al., 2007). The EVENT-experiments are located in the Ecological Botanical Garden of the University of Bayreuth, Germany (49°55'19"N, 11°34'55"E, 365 m asl). The experimental design consists of two factors: 1) extreme weather manipulation: drought (D), weekly average precipitation amounts (W), and ambient conditions for control (C), and 2) community diversity: *Holcus lanatus* (L.) monocultures (1-), *H. lanatus* growing together with one grass species (2-), with one grass species and two forbs (4-) or with one grass species and two forbs, with a legume species (4+) (Table 1). The setup consists of five replicates of each factorial combination. Communities were planted in 2 m x 2 m plots except for the monocultures that were grown in 1 m x 1 m plots. The factors were applied in a split-plot design, with community diversity nested within weather treatments. The species composition that had been planted was maintained by periodical weeding. Data acquisition was carried out within or close to the inner square meter of each plot to circumvent any edge effects.

Factor 1: Extreme drought event

Extreme drought (D) was induced using transparent rain-out shelters. Extreme greenhouse effects were avoided by starting the roof from a height of 80 cm, allowing air exchange near to the surface. The intensity of the drought was based on the local 1000-year extreme drought. Vegetation periods (March to September) from the years 1961-2000 acted as a reference period. Gumbel I distribution was fitted to the annual extreme, and a 1000-year recurrence was calculated (Gumbel, 1958). Drought was defined as the number of consecutive days with a daily amount of less than 1 mm precipitation. This resulted in a drought period with a length of 42 days that started on May 20th 2008 (day of the year (doy) 141) and ended on June 30th 2008 (doy 182). To end the drought treatment, we irrigated one third of the long-term weekly average amount of precipitation (see below) on three days in the week after drought, starting directly on the day after drought if no natural precipitation occurred (rewetting period).

The ambient control treatment (C) remained under natural conditions without any manipulation. To obtain an additional comparison with long-term mean conditions, we installed

regularly watered plots (Weekly Average, W). Here, the long-term weekly average amount of precipitation (vegetation periods 1961-2000 as a reference) minus the naturally occurring precipitation during that particular week was irrigated once per week, to ensure continuous water availability. Figure 1 provides data on soil moisture, precipitation and temperature at the time that measurements were taken.

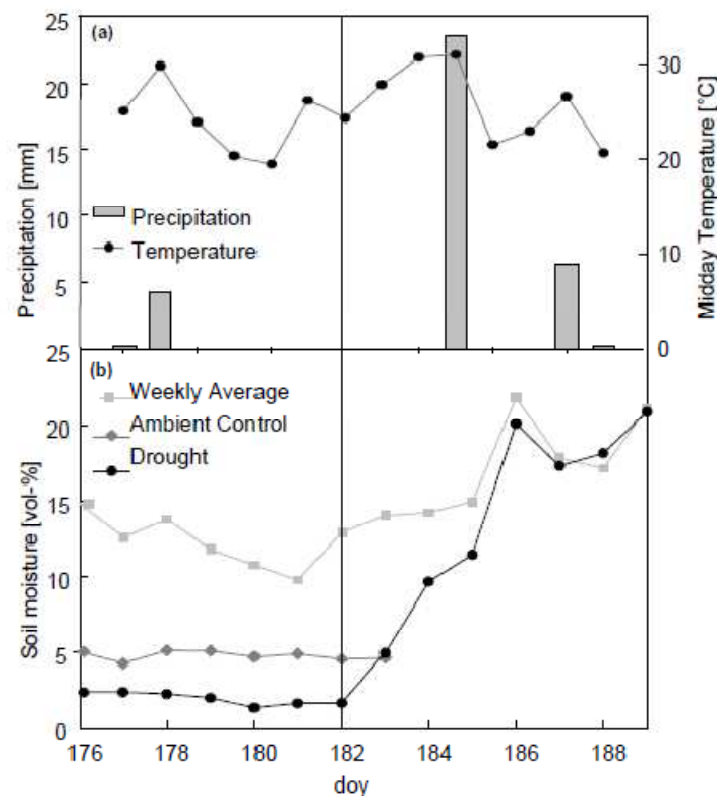


Figure 1 Natural precipitation (a), midday temperature (a) and soil moisture (b) data within the EVENT-experiment over the measuring period (last week of drought treatment and first week of post-drought recovery phase). Vertical black line shows the end of the drought treatment on the day of the year (doy) 182. Soil moisture was measured in four-species communities without legume species at a depth of 5 cm using FD-sensors, the means of hourly readings for each day are given. Ambient Control (C) sensors stopped on day 183 (due to technical problems).

Factor 2: Plant communities

All mixed plant communities consist of 100 individuals per 2 m x 2 m plot. All individuals were planted in a regular grid 20 cm apart from neighbouring individuals. All plants were pre-grown from seeds in autumn 2004 and planted outside in April 2005. Monocultures (1 m x 1 m plots, 25 individuals) were installed in autumn 2006 with plants that were planted outside

since April 2005. Thus, individuals were three years old during the measurements. The target species *H. lanatus* grew in four community compositions, differing by the number of species (one to four) and the number of functional groups (one to three) (Table 1).

Table 1 Community composition and diversity levels for communities with the target species *Holcus lanatus*.

community	name	functional groups	species
monoculture	1-	one grass	<i>Holcus lanatus</i>
Two-species community	2-	two grasses	<i>Holcus lanatus</i> <i>Arrhenatherum elatius</i>
Four-species community without a legume	4-	two grasses two forbs	<i>Holcus lanatus</i> <i>Arrhenatherum elatius</i> <i>Plantago lanceolata</i> <i>Geranium pratense</i>
Four-species community with a legume	4+	two grasses one forb one legume	<i>Holcus lanatus</i> . <i>Arrhenatherum elatius</i> <i>Plantago lanceolata</i> . <i>Lotus corniculatus</i>

Response parameters

Leaf water potential

Leaf water potential (LWP) was determined using a PMS 600 pressure bomb (PMS Instrument Company, Albany, USA). It was measured before dawn on June 28th, July 3rd and July 5th (doy 180, 185 and 187), treatment ended on June 30th (doy 182). We also performed midday measurements on June 25th (doy 177) and July 3rd (doy 185). Leaves were cut with a razor blade, transported to the pressure bomb in an aluminium foil bag and measured immediately. LWP was the negative applied pressure in MPa when xylem-sap was visible at the cut end of the leaf.

Chlorophyll a fluorescence

Non-invasive chlorophyll *a* fluorescence measurements were used to measure photosynthetic performance, namely the quantum efficiency of photosystem II as described by Rascher et al. (2000). We measured predawn fluorescence of dark-adapted leaves between 0130 hrs and 0400 hrs during the last week of the drought treatment on June 26th (doy 178) and on three

days during the recovery phase after drought (June 3rd (doy 185) three days after rewetting; June 5th (doy 187) five days after rewetting and June 7th (doy 189) 7 days after rewetting). The maximum quantum efficiency of photosystem II (F_v/F_m) was derived from the maximum fluorescence of the dark-adapted leaf after applying a saturating light pulse (F_m) and the variable fluorescence yield of the dark adapted leaf ($F_v = F_m - F_0$) with F_0 being the steady state fluorescence yield of the dark adapted leaf (Maxwell & Johnson, 2000). Chlorophyll *a* fluorescence was obtained using a pulse-amplitude-modulated photosynthesis yield analyzer (PAM 2000 by H. Walz GmbH, Effeltrich, Germany) with a leaf clip holder as described by Bilger et al. (1995).

As we wanted to obtain the species response of *H. lanatus* within the community, measured individuals were chosen randomly on each day measured, thus representing the overall performance of the species within the community, and not the performance of one individual. A fluorescence standard was measured prior to each plot, which was then used to normalize the fluorescence values that were obtained. We measured only fully expanded leaves in the upper third of each plant individual and used the leaves of four plant individuals per plot. To determine F_v/F_m the median of all data within one plot was taken for analysis to avoid pseudo-replication. We measured the chlorophyll *a* fluorescence of *H. lanatus* under drought conditions in all communities. Under ambient conditions (C) we only conducted fluorescence measurements in monocultures (1-) and in communities with a legume species (4+). Under conditions with a weekly average precipitation (W) we carried out measurements in two-species communities (2-) and four-species communities without a legume species (4-). (Neither monocultures nor four-species communities with a legume species were available).

For statistical analysis, C (ambient conditions) and W (weekly average precipitation) were treated as two different manipulations. However, significant differences in F_v/F_m between the ambient control (C) and the long-term control (weekly average precipitation, W) only occurred on July 3rd (doy 185, 3 days after rewetting). On doy 185 individuals growing under ambient conditions in monocultures showed significantly higher F_v/F_m than for those growing in the controls of all other communities, and were even higher than in communities which regularly received weekly average amounts of precipitation. There were no further differences between the ambient control (C) and the long-term control (W) in different communities at any other time of measurement, therefore, both ambient conditions (C) and weekly average precipitation (W) are referred to as “control” in the following.

Pigment analysis

Leaf samples for pigment analysis were taken on the last day of the drought treatment (June 30th, day 182). 3 cm² of leaf material from one leaf per plot were cut and stored immediately in liquid nitrogen until they could be transferred to -80°C. Leaf discs were ground in a mortar in liquid nitrogen with Krytobalit to improve cell lysis, and pigments were extracted using 100% ice-cold acetone. The content of chlorophyll *a*, chlorophyll *b* and carotenoids were determined photometrically at three different wavelengths (470 nm, 645 nm, 662 nm) (Lichtenthaler, 1987).

Nitrogen concentrations and $\delta^{15}\text{N}$ natural abundance in 2007 and protein content in 2008

To determine $\delta^{15}\text{N}$ natural abundance [‰] and total nitrogen concentration [%] of leaves of *H. lanatus*, leaf material was sampled in 2007 at the end of the drought treatment. To determine isotope ratios and N concentrations, 1 ± 0.1 mg of dried and finely ground plant material was weighed into tin capsules and analysed using an elemental analyser (EA 3000, EuroVector, Italy). Resulting gases out of Dumas Combustion were dried and separated using a GC-column coupled online to a ConFlo III interface (Thermo Electron, Bremen, Germany) connected to an isotope-ratio mass spectrometer (MAT 253, Thermo Electron, Bremen, Germany) (Gehre *et al.* 1994).

We used the ratio of $^{15}\text{N}/^{14}\text{N}$ in the sample and a standard (atmospheric N_2) to determine the $\delta^{15}\text{N}$ natural abundance value of plant leaves (Mariotti, 1983, Shearer & Kohl, 1986). The $\delta^{15}\text{N}$ values were calculated as follows:

$$\delta^{15}\text{N} [\text{‰}] = (\text{R}_{\text{sample}}/\text{R}_{\text{standard}} - 1) * 1000,$$

where R represents the ratio of $^{15}\text{N}/^{14}\text{N}$ isotopes. The N concentration of samples was calculated in comparison to a reference material with known N-concentration. The experimental design did not differ between 2007 and 2008, but in 2007 the treatments were applied according to the 100-year-extreme event, so drought lasted only for 32 days.

In 2008, leaf material was sampled on the last day of drought treatment, to determine protein-bound amino acids. Mixed samples of all plots were frozen in liquid nitrogen until transfer to -80°C and freeze-dried. Amino acids of the protein fraction were extracted. Amino acid concentrations were measured with an ion exchange chromatograph (Biotronik, amino acid

analyser LC 3000) and protein content was calculated by pooling the content of each amino acid in the protein fraction. Weiner et al. (2010) summarize the applied method in detail.

Net productivity per individual and community productivity

Four plant individuals in the inner square meter of the 2-, 4- and 4+ communities were marked at the beginning of the vegetation period. Complete aboveground biomass of these individuals was harvested one week after the drought treatment had ended, on July 10th to calculate the mean aboveground net productivity per individual (NP_{ind} [g/tussock]). In monocultures, all individuals were harvested and counted to calculate NP_{ind} . To determine community productivity, all plant material of the inner square meter of each plot was cut. Harvested plant material was dried for 48 hours at 70°C and weighed.

Statistical analysis

We examined residuals versus fitted plots and normal qq-plots prior to each analysis to test whether the assumptions for ANOVA, homogeneity of variances and normality could be met (Faraway, 2006). If this was not the case, data were power- or log-transformed.

To test for significant differences in NP_{ind} and LWP, we performed a two-factorial ANOVA. We corrected for the split-plot-design by specifying the nesting of community composition within the treatment in the Error-Term of the mixed model. Fixed factors were community composition and weather manipulation. To more closely examine the community effects on *H. lanatus* individuals during drought, multiple comparisons were performed for linear mixed effect models with Tukey correction.

Fluorescence data and pigment data were analyzed differently: Although control (C) and regular watering (W) did not show significant differences on four out of five days of measurements, the effect of weather manipulation and community composition were analyzed separately here. The effects of community composition within drought treatments were examined using multiple comparisons for linear mixed effect models with Tukey correction and the effects of weather manipulation within one community composition were analyzed in a multiple one-way ANOVA using linear models.

All statistical analyses were performed using R (R Development Core Team 2006). For mixed effect models we used the software package *nlme* (Pinheiro et al., 2008), and for multiple Tukey comparisons with mixed models we used the software package *multcomp* (Hothorn *et al.*, 2008).

RESULTS

PERFORMANCE OF *H. LANATUS* DURING THE DROUGHT

Leaf water potential

Both the midday leaf water potential (LWP), measured on June 25th (doy 177, Fig. 2a) after 37 days without precipitation, and the predawn water potential, measured on June 28th (doy 180, Fig. 2b) after 40 days without precipitation, showed highly significant differences between drought treated and control plants (doy 177: $P=0.0065$; doy 180: $P=0.0001$). The drought effect was consistent for all plant communities (Fig. 2a, b).

Community composition affected predawn LWP on doy 180 (Fig. 2b): *H. lanatus* growing in four-species communities with a legume species (4+) showed a significantly more negative LWP than *H. lanatus* growing in four-species communities without a legume species (4-) (Tukey test: $P<0.001$) and monocultures (Tukey test: $P<0.001$). LWP in *H. lanatus* was significantly lower in two-species communities than in monocultures (Tukey test: $P<0.001$) and marginally lower than in the four-species communities without a legume (Tukey test: $P=0.055$).

Maximum quantum efficiency

Species composition significantly affected the maximum quantum efficiency (F_v/F_m) of dark-adapted leaves of *H. lanatus* after 38 days of simulated drought (doy 178: $P<0.0001$, Fig. 2c). F_v/F_m in four-species communities with a legume species (4+) was significantly lower than at the other three diversity levels (Tukey test, $P<0.0001$, Fig. 2c) whereas no differences occurred between all other communities without the legume species (1-, 2- and 4-). Accordingly, treatment effects were tested separately for every community with corresponding controls. *H. lanatus* individuals growing in monoculture (1-) and in four-species communities with and without a legume species (4- and 4+) showed significant drought effects on F_v/F_m compared to controls on doy 178 (1-: $P=0.001$; 2-: $P=0.175$; 4-: $P=0.011$; 4+: $P=0.007$, Fig. 2c).

Leaf pigment content

Visual inspection revealed severe signs of pigment and chlorophyll degradation in *H. lanatus* growing in the communities that included a legume species under drought stress, as leaves appeared yellowish and wilted. This could not be confirmed by a pigment analysis, as neither drought, nor community composition during drought had a significant effect on the content of chlorophyll *a*, chlorophyll *b*, the overall carotenoids or the total photosynthetic pigment content (Fig. 2d) in leaves of *H. lanatus*. Albeit a trend to lower total pigment content existed in leaves of *H. lanatus* growing in both four-species communities in the drought treatment (Fig. 2d), no statistically significant differences occurred.

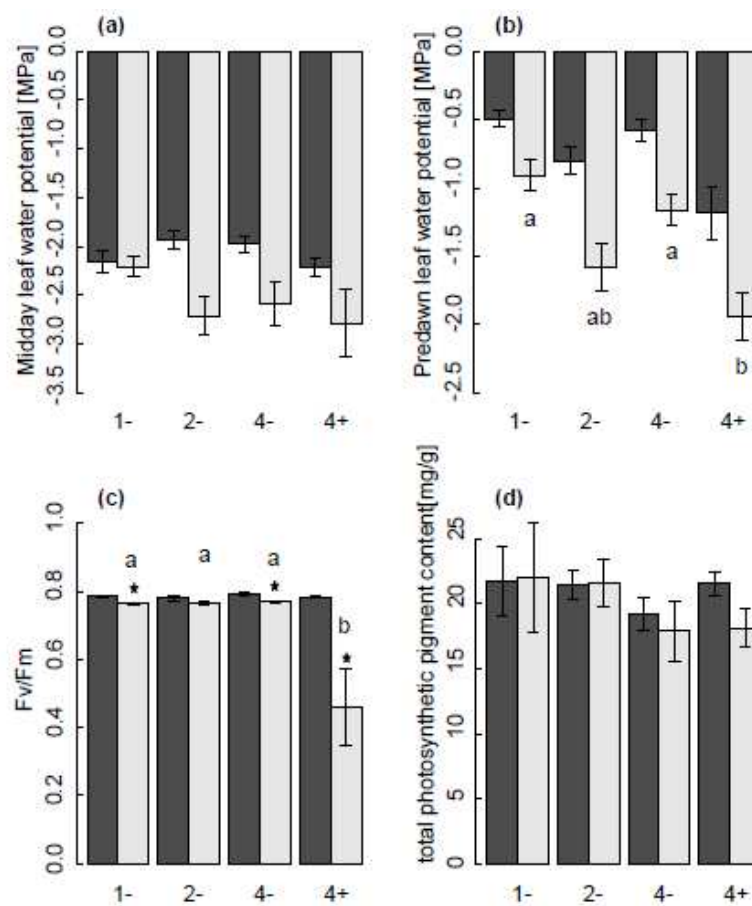


Figure 2 Performance of *Holcus lanatus* in control treatments (dark-grey bars) and drought treatments (light-grey bars) characterized by four different parameters: (a) midday leaf water potential (LWP) (day of the year (doy) 177) and (b) predawn LWP (doy 180) – both of which showed a significant overall drought effect, (c) maximum quantum efficiency (F_v/F_m) and (d), total pigment content. Error bars indicate 1 standard error of the mean. Asterisks indicate significant differences *between* drought and the corresponding control ($P < 0.05$). Different letters indicate significant differences between the species compositions *within* drought treatment ($P < 0.05$).

Nitrogen concentration, $\delta^{15}\text{N}$ and protein content

In 2007, N concentration [%] was significantly decreased in drought plots compared to ambient control ($P < 0.0001$) and *H. lanatus* from different communities revealed different N concentrations ($P < 0.0001$). *H. lanatus* from monocultures and two-species communities had significantly higher N concentrations in its leaf tissue than *H. lanatus* from four-species communities without a legume species (Table 2). The isotopic composition of N ($\delta^{15}\text{N}$, Table 2) did neither differ between treatments (ANOVA: $P = 0.9$), nor between communities (ANOVA: $P = 0.1$).

Table 2 Nitrogen concentrations and $\delta^{15}\text{N}$ values in leaves of *Holcus lanatus* at the end of the 100-year extreme drought in 2007 measured under ambient conditions (control = C) and in the drought treatment (D). Means \pm 1 standard error of the mean are given (n = 5).

		community			
		1-	2-	4-	4+
N [%]	C	2.1 \pm 0.027	2.0 \pm 0.001	1.7 \pm 0.009	1.8 \pm 0.000
	D	1.6 \pm 0.003	1.6 \pm 0.006	1.2 \pm 0.001	1.3 \pm 0.003
$\delta^{15}\text{N}$ [‰]	C	-2.09 \pm 0.24	-1.98 \pm 0.2	-2.69 \pm 0.41	-1.82 \pm 0.06
	D	-2.03 \pm 0.04	-1.02 \pm 0.24	-2.52 \pm 0.50	-2.58 \pm 0.39

Drought significantly decreased the protein content in 2008. Protein content was lowest in four-species communities without a legume species and highest in two-species-communities (Fig. 3). Protein content differed significantly for *H. lanatus* between plants from four-species communities without a legume species compared to plants from monocultures and two-species communities (Tukey test: 4-vs.2-, $P < 0.001$; 4-vs.1-, $P = 0.048$) and between plants from four-species communities with a legume species and plants from two-species communities ($P = 0.003$).

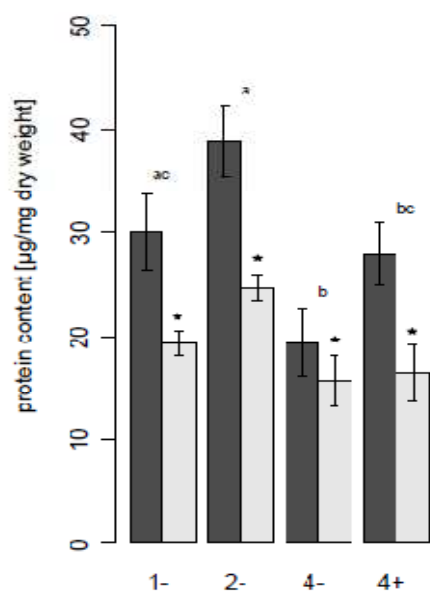


Figure 3 Total protein content in leaves of *Holcus lanatus* at the last day of drought period in the control (dark-grey bars) and drought treatment (light-grey bars). Error bars indicate 1 standard error of the mean. Asterisk indicate significant differences between drought and control ($P < 0.05$), different letters indicate significant differences in overall community response.

RECOVERY OF *H. LANATUS* AFTER THE DROUGHT

Leaf water potential

On the third day of recovery after the drought (doy 185), predawn LWP of *H. lanatus* was still significantly reduced compared to the control ($P=0.03$, Fig. 4a). LWP during recovery was generally not affected by species composition, but the magnitude of the LWP reduction still tended to be more negative in four-species communities with a legume species (4+) as well as in the two-species communities (2-) for predawn measurement on doy 185 (Fig. 4a). For midday measurements on doy 185 and 187 neither a treatment effect nor a composition effect occurred (Fig. 4b, c).

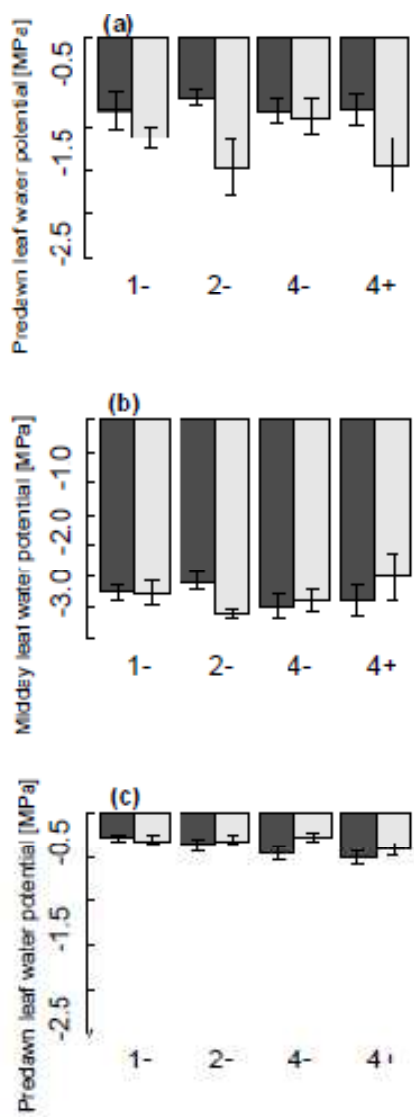


Figure 4 Leaf water potential (LWP) of three measurements in the post-drought recovery phase in *Holcus lanatus* measured in the control (C, dark-grey bars) and drought treatments (D, light-grey bars). (a) Predawn LWP three days after rewetting showed a significant overall drought effect. (b) Midday LWP three days after rewetting, and (c) predawn LWP five days after rewetting showed no more significant effects. Error bars indicate 1 standard error of the mean.

Maximum quantum efficiency

The drought treatment had a prolonged effect on F_v/F_m in leaves of *H. lanatus* individuals within monocultures in the first week of the recovery phase, taking into account treatment differences in the communities separately (1-: D versus C: July 3rd/doy 185: $P=0.004$; July 5th/doy 187: $P=0.025$; July 7th/doy 189: $P=0.043$; Fig. 5a, b, c). In two-species communities (2-) as well as in both four-species communities (4- and 4+), we found significant drought effects on F_v/F_m in *H. lanatus* only up to five days after rewetting, which was applied at June 30th/doy 182 (2-: D vs. W on July 3rd: $P=0.040$ and on July 5th: $P=0.0113$; 4-: D vs. W on July 3rd $P<0.001$ and on July 5th $P=0.003$; 4+: D vs. C on July 3rd $P=0.038$ and on July 5th $P=0.008$; Fig. 5a, b). Thus, treatment effects disappeared in all communities except for in the monocultures one week after the drought treatment had ended (Fig. 5c).

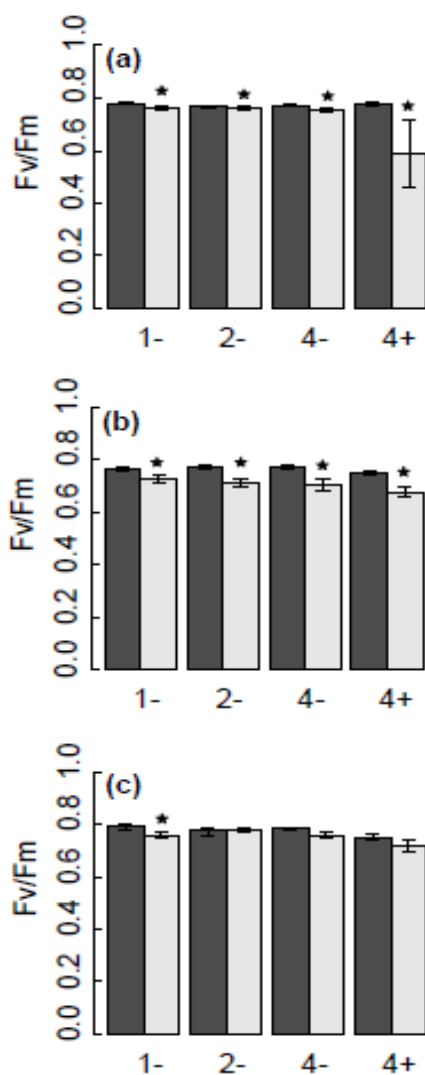


Figure 5 Maximum quantum efficiency (F_v/F_m) measured in the control (dark-grey bars) and drought treatments (light-grey bars) in the post-drought recovery phase three (a), five (b) and seven days (c) after rewetting. Error bars indicate 1 standard error of the mean. Asterisks indicate significant differences between drought and the corresponding control ($P < 0.05$).

Individual net productivity and community productivity

Previous drought had no effect on biomass production per individual (NP_{ind} , Fig. 6a), but community composition affected NP_{ind} , independent of the treatment ($P < 0.0001$): Individuals of *H. lanatus* growing in monocultures (1-) and two-species communities (2-) showed significantly larger NP_{ind} than individuals growing in both four-species communities (Tukey test 4- vs. 1- and vs. 2-: $P < 0.001$; 4+ vs. 1-: $P = 0.021$; 4+ vs. 2-: $P = 0.002$). NP_{ind} of *H. lanatus* in four-species communities with a legume species (4+) was significantly larger than NP_{ind} of *H. lanatus* in four-species communities without a legume species (4-) (Tukey test: 4- vs. 4+, $P = 0.0145$).

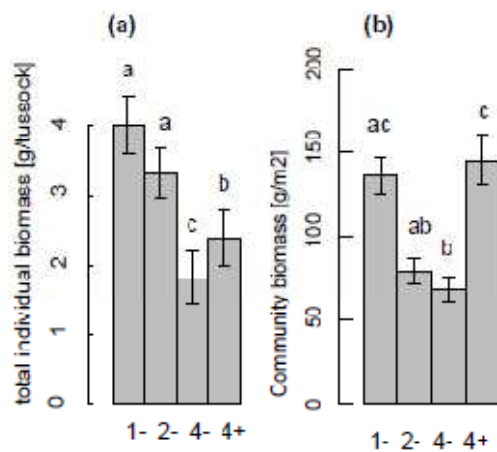


Figure 6 Biomass production (a) of *Holcus lanatus* individuals (NP_{ind}) in different communities and (b) of whole communities. Different letters indicate significant differences between communities which are monoculture (1-), two-species mixture (2-) and four-species mixtures without (4-) or with (4+) a legume species.

Drought had no effect on community productivity (Fig. 6b), but community productivity differed significantly between the different compositions ($P < 0.0001$). Most biomass was produced in four-species communities with a legume species, and least in four-species communities without a legume species.

DISCUSSION

The stress gradient hypothesis (Bertness and Callaway 1994) states, that facilitation becomes more important during stress. Furthermore, ecological theories predict that facilitation is one of the key mechanisms that ensure ecosystem stability in more diverse communities. We attempted to integrate both approaches by linking species-specific performance and community ecology. Therefore we investigated the vegetation response of one species growing in communities which differ in their composition and species number. We hypothesized that community diversity, the number of species and functional traits within a community, should have a positive effect on ecophysiological performance of the target species *Holcus lanatus*. Some studies have shown a facilitative effect of community diversity on single species performance (Callaway et al., 1997; Cardinale et al., 2002).

Buffering capacity of diversity during extreme stress

We observed reductions in predawn leaf water potential (LWP; Fig. 2b) in two-species communities and four-species communities with a legume species when compared to monocultures. The more negative the LWP is, the lower is the water availability a plant experiences (Larcher, 1994). Thus, predawn LWP results indicate that monocultures experienced the least water stress during the drought treatment which is also confirmed by the nearly unchanged midday LWP (Fig. 2a) for *H. lanatus* in monoculture. However, the maximum quantum efficiency of dark-adapted leaves (F_v/F_m ; Fig. 2c) was reduced in monocultures, indicating minor photoinhibition (Maxwell and Johnson, 2000).

The highest performance reductions during the drought period, revealed by strong negative effects on predawn LWP and F_v/F_m (Fig. 2b, c), were found for *H. lanatus* growing in communities with a legume species (4+). The outstandingly low values for F_v/F_m are most likely due to wilting or early senescence of *H. lanatus* in 4+ communities although the pigment data (Fig. 2d) only show a trend towards lower chlorophyll content (and no significant differences). However, visual inspection provided signs of severe pigment and chlorophyll degradation. The discrepancy between visual appearance and results from pigment analyses might be related to the sampling: whereas we “see” the overall species performance, we sampled only one leaf per plot for the pigment analysis ($n = 5$), which nonetheless only provides a snapshot of the overall species performance. Therefore, F_v/F_m ($n = 20$) may provides a more representative measure. The validity of the non-invasive F_v/F_m is

further corroborated by chlorophyll *a* fluorescence measurements in light-adapted leaves of *H. lanatus* in 2008 (data not shown) and in two previous years. In 2006 and 2007, the maximum electron transport rate (ETR_{max}), a parameter derived from the quantum yield of light-adapted leaves, in *H. lanatus* in 4+ communities exposed to 100-year drought events were significantly lower than in plants from monocultures (**manuscript 1**; personal communication Lea Martin). Overall, our ecophysiological results do not support the stress-gradient hypothesis: no increase in facilitation – but rather an increase in stress – occurred under an extreme drought event in the communities with a higher number of species and functional traits as it has been reported from studies in (semi-)arid habitats (see Maestre et al. 2004 and references within).

The role of Lotus corniculatus for facilitation during stress

The extreme drought effects on *H. lanatus* in 4+ communities with a legume species (*Lotus corniculatus*) are not in line with other studies that indicate a facilitative role of legumes for plant interactions in semi-natural mesic grassland habitats (Gosling, 2005; Palmberg et al., 2005; Temperton et al., 2007; Fornara et al., 2009). This result is also not in accordance with a greenhouse pot experiment conducted by Wurst & van Beersum (2009), where *H. lanatus* has outcompeted *L. corniculatus* over a drought period. However, most studies showing the facilitative role of legumes focus on community productivity, a long-term parameter. Community productivity is also facilitated by the presence of *L. corniculatus* in our study (Fig. 6b); mainly due to the highly productive legume itself (personal communication Kerstin Grant) but also due to higher individual biomass production of e.g. *H. lanatus* (Fig. 6a). The positive effect of legume presence on community biomass (Fig. 6b) is likely to lead to higher competition for water under extreme drought stress as reported for experimental grassland communities exposed to a naturally occurring drought event (Verheyen et al. 2008) and from controlled out-door heat/drought pot experiment with mesic grassland species (van Peer et al., 2004; Zavalloni et al. 2009). The combination of low LWP for drought treated *H. lanatus* in communities with a legume species (Fig. 2a, b) and the high community productivity of these communities (Fig. 6b) suggests that this mechanism also determines plant interactions during drought within this well-controlled field experiment. Our findings support studies which also show an increase of competition instead of facilitation under extreme water shortage (Tielbörger and Kadmon 2000; Ludwig et al. 2004; Lortie and Turkington 2008).

Under non-stress conditions, on the other hand, the presence of the legume species may have a positive effect on the N availability for neighbouring species as trends towards higher protein content (Fig. 4), higher individual biomass (Fig. 6a), higher N concentration and $\delta^{15}\text{N}$ values (Table 2) for *H. lanatus* plants in communities with a legume species (4+) compared to four-species communities without a legume species (4-) suggest. Low $\delta^{15}\text{N}$ values often indicate low N availability for plants and changes in $\delta^{15}\text{N}$ values of non-legume species towards zero can indicate facilitative interactions between a legume and a non-legume species by N transfer or N sparing (Högberg, 1997; Temperton et al. 2007). Drought stress does not only reduce water availability but can have profound negative effects on the N_2 -fixing ability of legumes (Serraj et al., 1999; Galvez et al., 2005). Thus, there might be additional negative effects in 4+ communities during drought. Additionally, data for protein contents in our study partly support these findings. Protein content of *H. lanatus* in all communities declined under drought (Fig. 4), but the drop in four-species communities with a legume species was larger (40%) than the decline in four-species communities without a legume species (20%). Furthermore, $\delta^{15}\text{N}$ values (Table 2), which were 33% higher in communities with a legume species (4+) compared to four-species communities without a legume species (4-) under ambient conditions (control), were even more reduced for *H. lanatus* in 4+ compared to 4- under drought. This indicates a loss of facilitative interactions (N transfer or N sparing) between the legume species and neighbouring plants. The unexpected strong decline in F_v/F_m for *H. lanatus* in 4+ could thus possibly be due to the lack of N_2 -fixation under drought, which might have caused a shortage in N-supply, particular in nitrate. CO_2 and nitrate are possible electron-acceptors for reduction equivalents from photochemistry. A sudden lack of nitrogen and nitrate availability together with a lack of CO_2 (caused by closure of stomata in response to drought) could cause an over-energetization and photodamage to photosystem II in leaves of *H. lanatus*. A reduction in F_v/F_m can therefore be a sign of persistent photodamage, as individuals are severely stressed and not able to recover over night. Additionally, a lack of nitrogen can prevent the rapid repair of photosynthetic proteins.

The role of species and functional diversity for post-drought recovery

Not only the magnitude of the effect during the stress, but also the speed of recovery after the stress period determine resilience and stress response and are thus important for facilitation research. Here, despite of showing extreme signs of stress compared to other communities during the drought treatment (Fig. 2), *H. lanatus* growing in communities with a legume

species (4+) made an unexpected rapid recovery: whereas F_v/F_m of *H. lanatus* in 4+ was lowest at the end of the drought treatment, it fully recovered within the following week and deviated no longer from those in the other communities (Fig. 5). One week after rewetting only the monocultures still showed negative effects of the applied drought treatment on F_v/F_m (Fig. 5), although LWP was highest during the treatment period (Fig. 2b), which suggests the lowest level of recovery. Data from chlorophyll *a* fluorescence measurements in light-adapted leaves (data not shown) confirmed the results from dark-adapted leaves and corroborate the high potential for recovery of *H. lanatus* in communities with the highest diversity of functional traits. The effect could be partly attributed to resprouting and the quick growth of new leaves and partly to a higher potential for facilitation and niche complementarity which increase the recovery of existing plant material. The net productivity of individuals (NP_{ind}) also supported these findings: *H. lanatus* individuals in communities with a legume species (4+) were more productive (although being subjected to greater stress levels; Fig. 2b, c) than in four-species communities without a legume species (4-).

Increased biodiversity often leads to an improvement in ecosystem productivity and resilience (e.g. Tilman and Downing, 1994; Isbell et al. 2009). In our study, species number had a positive effect on the recovery of individuals from a *H. lanatus* population (Fig. 5c), but effects of the presence of *Lotus corniculatus* in four-species communities were stronger than those of biodiversity. Van Ruijven et al. (2010) show in legume-free experimental grassland communities exposed to a naturally occurring extreme drought that diversity *per se* enhanced community recovery after drought, but not resistance. Our findings further emphasize the facilitative role that biodiversity, but in first order, legumes play under non-stress conditions in plant communities and also corroborate the role of nitrogen for complementarity effects (Fargione et al., 2007). Higher nitrogen availability through the presence of an N_2 -fixing legume species after the drought period can add to a more efficient recovery and performance of photosystem II, because nitrate can be used as well as CO_2 for reduction through reduction equivalents out of the photosynthetic light reaction.

CONCLUSIONS

To summarize, our measurements during the drought treatment neither directly support the stress gradient hypothesis nor the insurance hypothesis: we did not find better performance and increased facilitation in more diverse communities or communities with a legume species during an extreme drought event. These findings support studies which also show an enhance-

ment of competition instead of facilitation under extreme water shortage (Tielbörger et al., 2000; Ludwig et al., 2004). However, presence of a legume species did facilitate recovery: *H. lanatus* growing in communities with a legume species showed a quick recovery after severe drought stress whereas *H. lanatus* growing in monocultures showed reduced photosynthetic efficiency for the longest time period.

Our results indicate that functional diversity can enhance ecosystem stability and resilience and is more important for ecosystem functioning than diversity in terms of species richness (Scherer-Lorenzen, 2008). Our study therefore provides a link between community ecology and facilitation research: it is likely that better resilience and overall performance (due to legume presence and better resource availability) of the whole community helped to speed up recovery of the widespread grass species *H. lanatus*. Thus, our results also indicate that two prominent ecological hypotheses, namely the insurance hypothesis and the stress gradient hypothesis, albeit making predictions on different organisation levels (species vs. community performance), can be brought together. Therefore, more detailed studies that focus on isolating direct and indirect facilitation, and sampling or complementarity effects (Fridley, 2001; Loreau et al., 2001) are needed to investigate the role of community diversity and in particular that of legume presence for facilitation.

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MANUSCRIPT 3

THE USE OF THE $\delta^{15}\text{N}$ NATURAL ABUNDANCE METHOD TO ASSESS
FACILITATION AND RESTORATION SUCCESS IN CALCAREOUS
GRASSLAND

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ABSTRACT

Facilitation, in the form of extra nitrogen input into ecosystems by nitrogen-fixing plants, has received very little attention in degraded systems in need of restoration. We investigated whether positive interactions between plants would increase with the severity of the abiotic environment in a calcareous grassland restoration project on ex-arable land. The restoration treatments consisted of topsoil removal and hay transfer (from a reference grassland site).

We used the $\delta^{15}\text{N}$ natural abundance method to assess facilitative interactions between pairs of species across an environmental nutrient and water availability gradient. Plant pairs, N_2 -fixing legume species and their non-fixing neighbors, were either adapted to calcareous grasslands (target species) or mesic grasslands (non-target species). We found that restoration treatments were reflected in the $\delta^{15}\text{N}$ signal of the non-legume species: increasing restoration effort led to significantly decreasing $\delta^{15}\text{N}$ values. Functional group identity as well as species identity affected the $\delta^{15}\text{N}$ signal. We found only weak evidence for N-facilitation (using the $\delta^{15}\text{N}$ method), with abiotic soil N dynamics overriding any potential facilitative signal from neighboring legume species. Although the $\delta^{15}\text{N}$ method could not be used in this calcareous grassland to clearly assess facilitation, this work has highlighted the potential of using the integrative character of the $\delta^{15}\text{N}$ signal in plants to provide a useful tool for evaluating restoration success (transformation from eutrophic to oligotrophic systems).

Keywords

chalk grassland, facilitation, legumes, positive plant-plant interactions, restoration, stable isotopes

N-parameters

$\delta^{15}\text{N}$: ratio of the heavier (^{15}N) over the lighter (^{14}N) stable isotope of nitrogen in plant or soil samples (see eq. 1); $\Delta\delta^{15}\text{N}$: foliar $\delta^{15}\text{N}$ values standardized by background bulk soil $\delta^{15}\text{N}$ values (see eq. 2); %Ndfa: percent nitrogen derived from atmosphere in N_2 -fixing species (see eq. 3), N_{min} : mineralized N-forms in soil solution, both nitrate (NO_3^-) and ammonium (NH_4^+) together

INTRODUCTION

Low-productive grasslands with a highly specialized set of species, such as calcareous or dry acidic grasslands, form important cultural landscapes of high conservation value in Europe (Riecken et al., 1997; Isselstein et al., 2005; Kiehl & Pfadenhauer, 2007). Due to agricultural intensification and land-use changes, the restoration of low-productive, species-rich calcareous (or species-poor acidic) grasslands has become an important tool in conservation and ecological restoration in Central Europe. So far, the role of facilitation in the successful restoration of semi-natural species-rich grasslands has rarely been studied in Europe (e.g. Ryser, 1993). Facilitation is defined as the ability of one species to modify the environment beneficially for another species (Connell & Slatyer, 1977), whereby one species has a positive effect on *neighboring* or *subsequent* species. The most prominent example for facilitation is that of nurse-plant effects on neighbors, whereby a resident plant (mostly a shrub or cushion plant) enables a seedling of a different species to establish underneath it, thereby providing shelter (against grazing or climatic effects), nutrients or water. Positive nurse-plant effects have generally been found in environmentally extreme ecosystems such as arid (Pugnaire et al., 1996) or alpine/arctic environments (Arredondo-Nunez et al., 2009), but also in more mesic habitats (Ryser, 1993; Smit et al., 2007).

Equally important but less studied in either natural or semi-natural ecosystems, is facilitation by nitrogen-fixing legume species, which can have strong effects on nitrogen dynamics and productivity of non-fixing neighbors or subsequent species. For economic reasons two-species interactions (between N₂-fixing and neighboring species) have often been studied in intensive agriculture. Enhanced productivity and N-availability has been reported for pasture farming (McNeill & Wood, 1990b; Elgersma et al., 2000), crop rotation (Varvel & Wilhelm, 2003) and intercropping systems (Fujita et al., 1992; Li et al., 2007), including information about the relationship between the amount of N transferred and the distance between species or climatic and edaphic conditions (e.g. Giller & Cadisch, 1995). Only little is known, however, about N-dynamics and positive interactions between N-fixing and non-fixing species in more diverse plant communities. We know from biodiversity experiments in mesic grasslands that N-facilitation often contributes to positive biodiversity effects on community productivity and nutrient cycling. This is reflected in the N-status and often in the $\delta^{15}\text{N}$ value of non-fixing neighbors (Mulder et al., 2002; Spehn et al., 2002; Temperton et al., 2007; Carlsson et al., 2009). Temperton et al. (2007) found strong facilitative interactions between three different mesic grassland species (phytometers) and legume neighbors along a gradient of plant species

diversity in a field experiment. They found that legume species presence (but interestingly not abundance) affected plant N-concentration, N-content as well as $\delta^{15}\text{N}$ signals in neighbors, but also that an increasing number of surrounding species decreased N-concentration and the $\delta^{15}\text{N}$ signal.

The beneficial effect of legume species on neighbors is related to two overall mechanisms: transfer of nitrogen from legumes to neighbors (either via exudation, rhizodeposition and/or decomposition) and sharing of soil N-pools (an effect known as N-sparing (e.g. McNeill & Wood, 1990a): the legume species relies more on atmospheric N, thus increasing soil N-availability for the neighbors). Separating these two mechanisms requires one to be able to trace the movement of fixed atmospheric N_2 from the benefactor to beneficiary plant during facilitation and to assess soil N pools. The path of fixed nitrogen from benefactor to beneficiary plant can be followed using the stable ^{15}N isotope of nitrogen, which is heavier than the much more abundant ^{14}N isotope. The $\delta^{15}\text{N}$ natural abundance signal in plants is *per se* a function of the $\delta^{15}\text{N}$ values of the N-sources of the plant (Handley & Raven, 1992) and thus functions as an integrator of N-dynamics in a system (Robinson, 2001).

Högberg (1997) reviewed the topic of $\delta^{15}\text{N}$ natural abundance in plants and soils comprehensively and describes a variety of factors affecting the $\delta^{15}\text{N}$ signal in soil-plant systems (e.g. species identity, mycorrhiza, soil moisture, pH, N-status etc.). The $\delta^{15}\text{N}$ natural abundance method (established by Amarger et al., 1979) can be used for estimating the percent of N derived from atmosphere (%Ndfa) in aboveground plant parts of N_2 -fixing species (Shearer & Kohl, 1986). Despite its effectiveness for assessing N derived from atmosphere, few studies so far have used the $\delta^{15}\text{N}$ natural abundance method to study facilitative legume-neighbor interactions; most studies applied an enriched tracer to the system (called the isotope dilution method). The $\delta^{15}\text{N}$ natural abundance method (compared to the isotope dilution method), however, enables species to be identified as N-fixers, ($\delta^{15}\text{N}$ signal around zero) or non-fixers ($\delta^{15}\text{N}$ significantly different to zero) using the natural $\delta^{15}\text{N}$ signal (Virginia & Delwiche, 1982; Högberg, 1997). Equally, legume presence in grassland plant communities can affect the $\delta^{15}\text{N}$ signal of neighbors (Mulder et al., 2002; Spehn et al., 2002; Temperton et al., 2007; Carlsson et al., 2009) such that non-legume neighbors in the vicinity of legumes have lower $\delta^{15}\text{N}$ signals (closer to zero) than plants of the same species growing without legume influence, allowing an estimate of N-transfer from legumes to neighbors.

Very few studies have investigated the role of N-facilitation by legumes in grassland environments with extreme conditions (e.g. low nutrient and water availability) and higher species diversity than in mesic grasslands (e.g. calcareous grasslands). The stress gradient hypothesis predicts an increase in positive interactions with increasing environmental stress (Bertness & Callaway, 1994). N-facilitation along a gradient of N-availability should therefore be strongest in a substrate, where N-supply is lowest and weaker than under more mesic conditions.

Restoration of low productivity habitats (e.g. calcareous grassland in Europe) on ex-arable land often requires the removal of nutrient-rich topsoil to recreate the appropriate soil nutrient-dynamics for the target communities (Kiehl et al., 2006; Kiehl & Pfadenhauer, 2007). Restoration sites, which include a gradient of abiotic environments, for example on sites with and without topsoil removal, have the potential to provide an ideal testing ground (Bradshaw, 1993) for the stress gradient hypothesis. In this study, we investigated the role of N-facilitation by legumes on their neighbors in species-rich, calcareous grasslands across a gradient of N (and water) availability in a restoration experiment near Munich, including restoration fields with and without topsoil removal and sites with and without hay transfer for the introduction of target species. Our aim was to test the following hypotheses within a restoration setting:

- i. The $\delta^{15}\text{N}$ natural abundance method can be used to show facilitation by legume species on non-fixing neighbors in a species-rich calcareous grassland (as is possible in experimental mesic grasslands).
- ii. As predicted by the stress gradient hypothesis, positive interactions between legume and neighboring non-legume species (reflected by $\delta^{15}\text{N}$ values and N-concentrations in leaves), will increase along a gradient of abiotic stress, i.e. with strongest facilitation found on topsoil removal sites with severe N-limitation.

To test these hypotheses we sampled pairs (legumes and neighbors) of stress-tolerant target species as well as mesic non-target species in the four different restoration treatments and measured the N-concentration and the $\delta^{15}\text{N}$ value in leaves of these species, as well as the soil nutrient availability and the $\delta^{15}\text{N}$ signal in the bulk soil.

MATERIALS AND METHODS

Restoration study area and experimental design

The study area consists of ex-arable fields in the vicinity of the nature reserve “Garching Heide” (48°18’N, 11°39’E, 469 m asl) north of Munich, Germany, which were converted in 1993 in the course of a large-scale restoration project to reestablish nutrient-poor, species-rich calcareous grasslands. It is located in the Munich gravel plain on pararendzina soil evolved from melt water sediments from the Würm glacial period, which had been used as arable fields since the beginning of the 20th century (Pfadenhauer et al., 2000; Kiehl & Pfadenhauer, 2007). Water-holding capacity of the nutrient-poor soils is low. The climate is humid-temperate with a mean annual temperature of 7.8°C, and total annual precipitation of ~865 mm (data by Deutscher Wetterdienst for Oberschleißheim and Haimhausen-Ottershausen, time period 1961-1990, DWD, 2009).

Restoration treatments (topsoil removal, hay transfer) were performed full-factorial on a large scale on two different restoration fields in 1993 (providing two experimental blocks: block 1 = field 506/508 and block 2 = field 519/520). Topsoil removal, to achieve nutrient reduction of the substrate, consisted of removal of 40 cm agricultural topsoil down to the calcareous gravel. Topsoil removal resulted in a strong reduction of the total N-content and the content of exchangeable P and K in the soil (Table 1a), whereas the fertile humus layer stayed intact on sites without soil removal. Hay transfer (of diaspore-rich undried hay from the nearby nature reserve “Garching Heide”) was performed to overcome dispersal limitation of calcareous grassland species (Kiehl et al., 2006). On sites without hay transfer the number and cover of calcareous grassland species (including many legume species) was much lower than on hay-transfer sites, even 13 years after start of the restoration (Table 1b).

Both restoration treatments (abiotic factor: topsoil removal, biotic factor: hay transfer) were applied in a full-factorial design giving four differently treated areas per block (sorted by decreasing restoration effort): topsoil removal areas with hay transfer (+r+h) and without hay transfer (+r-h); no topsoil removal areas with hay transfer (-r+h) and without hay transfer (-r-h). The -r-h areas thus represent the natural succession from old field to grassland and hence form a restoration control (i.e. no restoration treatment carried out). Since 1995, the -r areas were either grazed by sheep or mown annually in July/August and the +r areas were mown only occasionally to remove woody species as mowing was usually not possible due to low

biomass production (Pfadenhauer et al., 2000; Pfadenhauer & Kiehl, 2003). Different management types showed only minor effects on flora and fauna compared to the major treatments topsoil removal and hay transfer and thus can be neglected in this study as stated by Kiehl & Wagner (2006). The biotic environment experienced by plants sampled for this study is summarized in Table 1b.

Table 1 Abiotic and biotic characteristics of the “Garchinger Heide” restoration project with (a) values for abiotic soil properties (0-10 cm, summarized from Pfadenhauer & Kiehl, 2003; Kiehl, 2005) and (b) species cover and species richness (assessed at two different scales: permanent plots and total area per restoration treatment; summarized from Hummitzsch, 2007). Values are means with standard deviations, n = 10-20 from permanent plots (4 m²) for all parameters except “species richness per restoration treatment” where n = 2 from the 2 blocks/restoration fields.

(a)	With topsoil removal		Without topsoil removal	
Skeletal content (fraction >2 mm) [% dry weight]	84.5 ± 5.8		69.9 ± 0.3	
P ₂ O ₅ [mg/100g]	4.1 ± 1.8		43.8 ± 5.7	
K ₂ O [mg/100g]	9.1 ± 4.2		58.1 ± 7.6	
N _{total} [%]	0.2 ± 0.1		0.43 ± 0.04	
C _{org} [%]	0.9 ± 0.6		4.6 ± 0.5	
C:N	10.1		10.6	
pH	7.2 ± 0.1		6.9 ± 0.1	

(b)	With topsoil removal		Without topsoil removal	
	With hay (+r+h)	Without hay (+r-h)	With hay (-r+h)	Without hay (-r-h)
Vegetation height [cm]	11.4 ± 5.0	14.1 ± 5.2	52.5 ± 7.3	58.3 ± 9.0
Cover [%]				
Litter	2.8 ± 1.3	2.4 ± 1.2	43.8 ± 24.1	30.0 ± 9.7
Bare soil	24.2 ± 13.6	74.8 ± 11.0	1.1 ± 2.3	1.5 ± 1.3
Vascular plants	48.4 ± 12.0	20.0 ± 14.2	85.7 ± 9.2	84.8 ± 5.8
Sum of cover	50.8 ± 13.7	26.7 ± 11.5	131.5 ± 15.0	118.5 ± 12.3
Target GL species	50.0 ± 14.0	22.2 ± 12.9	95.9 ± 15.1	35.5 ± 23.6
Mesic GL species	0.5 ± 0.4	3.3 ± 4.2	34.3 ± 11.1	77.4 ± 26.5
Ruderal species	0.3 ± 0.5	1.3 ± 1.6	1.3 ± 1.3	5.6 ± 3.7
Legume species	18.5 ± 6.8	5.9 ± 8.1	19.4 ± 6.5	12.4 ± 10.1
Forb species	30.7 ± 9.6	17.9 ± 4.6	46.0 ± 12.8	40.7 ± 10.6
Grass species	1.6 ± 1.6	2.3 ± 1.9	66.2 ± 10.5	65.3 ± 11.4
Species richness				
Total	23.1 ± 4.2	20.3 ± 6.9	27.9 ± 5.1	24.0 ± 3.6
Target GL species	21.1 ± 3.8	15.2 ± 4.0	19.1 ± 5.1	9.5 ± 3.3
Mesic GL species	1.5 ± 1.6	2.7 ± 2.6	7.5 ± 1.4	11.6 ± 1.8
Ruderal species	0.6 ± 0.6	2.4 ± 2.6	1.2 ± 0.6	2.9 ± 1.0
Species richness per restoration treatment				
Total	69.5 ± 4.9	85.0 ± 12.1	71.3 ± 1.8	78.7 ± 2.8
Target GL species	52.5 ± 2.7	51.0 ± 2.2	43.7 ± 1.8	34.3 ± 3.9
Mesic GL species	6.5 ± 0.6	9.5 ± 3.8	17.7 ± 1.8	22.0 ± 1.5
Ruderal species	10.5 ± 2.7	23.5 ± 4.9	10.0 ± 2.6	22.7 ± 2.7

Restoration treatments: with (+) or without (-) topsoil removal (r) and hay transfer (h)

Target species = calcareous grassland species (class Festuco-Brometea), GL = grassland

Sampling design

We collected samples in both restoration fields (block 1 and 2) which both contain all four restoration treatment combinations of topsoil removal and hay transfer (+r+h, +r-h, -r+h, -r-h). Plant and soil material were sampled along a 20 m long transect in the middle of each area to obtain representative samples for a given treatment.

In August 2007, we sampled plant material (1-2 individuals) at eight measuring points (equally distributed along the 20 m transects) or within a distance of maximum 2 m perpendicular to the transect if species did not occurred close by the transect line. We used every individual plant sample as a replicate (Table 2, n = (2) 6-16 per species and restoration treatment). The study was conducted at landscape scale on large restoration fields of several hectares and thus, according to Oksanen (2001), replications per area can be considered as independent samples in statistical analyses and not as pseudoreplicates *sensu* Hurlbert (1984). Due to the large-scale restoration approach, micro-climate of restoration sites was undisturbed by edge-effects, which often occur on small plots and undesired between-treatment dispersal (Pakeman et al., 2002) of introduced plants could be avoided (Pfadenhauer & Kiehl 2007).

Table 2 Overview of the number of plant pairs (non-legume species and their legume neighbor), control plants (non-legume species without legume neighbor) and legume species without non-legume neighbor sampled along transects in different restoration treatments.

Block	Transect	Treatment	Mesic grassland species				Target species			
			tri +	lot +	gal	lot	ant +	dor +	hel	ant
Block 1	T1	-r-h	8	8	6					6
	T2	-r+h		9			9			
	T3	+r+h					8	8	5	
	T4	+r-h				8	8	2	8	
Block 2	T5	-r-h	8	8	6					
	T6	-r+h		8	8					
	T7	+r+h					8	8	6	
	T8	+r-h		8	8		8		8	

Restoration treatments: with (+) or without (-) topsoil removal (r) and hay transfer (h)

Target species = calcareous grasslands species: ant = *Anthyllis vulneraria* (legume species), dor = *Dorycnium germanicum* (legume species), hel = *Helianthemum nummularium* (non-legume species) and mesic species: lot = *Lotus corniculatus* (legume species), tri = *Trifolium pratense* (legume species), gal = *Galium mollugo* (non-legume species)

We collected leaves of legume - non-legume pairs (<10 cm distance between each other) and control plants of the non-legume species (>30 cm distance to the next legume species) - so

non-legume species were obtained from different neighborhoods. As far as possible we collected pairs (legume/non-legume) of stress-tolerant target species (typical for calcareous grasslands of the class Festuco-Brometea) as well as pairs of non-target species (typical for mesic grasslands of the class Molinio-Arrhenatheretea) in the vicinity of the transect in all restoration treatments. The vegetation cover in the +r-h areas in both blocks was so low (Table 1b) that leaf samples had to be collected on the whole area and not along transects.

In practice in the field, since legume species abundance was high, control plants with an adequate distance to any legume species were very hard to find and were mostly sampled in larger distance from the transect. If it was not possible to collect plant pairs because of a missing non-legume partner, we sampled the legume species alone (Table 2).

Stress-tolerant target species sampled were: *Anthyllis vulneraria* L., *Dorycnium germanicum* (Gremli) Rikli (two legume species) and *Helianthemum nummularium* (L.) Mill. (small shrub); mesic, non-target species sampled were: *Trifolium pratense* L., *Lotus corniculatus* group (two legume species) and the forb *Galium mollugo* (Oberdorfer, 2001). Thus, both non-legume species were sampled in three different neighborhoods: non-legume species as neighbor of “legume A”, as neighbor of “legume B” and as “control plant (without legume neighbor)”. Although collection of control plants unaffected by legume vicinity was difficult, we nonetheless sampled control plants of *H. nummularium* and *G. mollugo* (>30 cm distance to any legume individual) and analyzed them separately for effects of legume neighborhood on their N status (see Table 2 for neighborhood combinations of species).

In November 2008, mixed soil samples from the upper 0-15 cm were taken at four positions along each transect for analysis of $\delta^{15}\text{N}$ and other abiotic parameters in the bulk soil. At each position soil material from five corings within a 2 x 2 m square was used for one mixed soil sample. Together with the soil, we collected root samples randomly (without species identification) to obtain approximate $\delta^{15}\text{N}$ and N [%] values for the belowground compartment of the vegetation and for mycorrhiza staining for estimation of mycorrhizal colonization (soil and root: n = 2 per restoration treatment). Legume roots, identified by detection of visible nodules, were present in every root sample.

Sample analysis

Plant samples were oven dried for 60 hours at 60°C and ground to fine powder using a Retsch ball mill MM 301 (Retsch GmbH, Haan, Germany) with stainless steel devices. Soil samples were sieved with a Retsch sieve with a pore size of 2 mm to homogenize the substrate and exclude large organic compounds and stones. An aliquot of the sieved soil was oven dried at 30°C for 72 hours and ground to fine powder with a Retsch ball mill using tungsten carbide devices. Ground samples were used for N-concentration and $\delta^{15}\text{N}$ (both plant and soil) and for P-concentration analysis (soil only). An aliquot of fresh soil was used for analysis of mineralized soil N (N_{min} : plant available NH_4^+ and NO_3^-).

For analyses of $\delta^{15}\text{N}$ natural abundance signal [‰] and N-concentration [%], ground plant or soil material was packed in tin capsules and measured using an element analyzer coupled with an isotope ratio mass spectrometer (EA-IRMS; EA = EURO-EA 3000 by HEKAtech GmbH, Wegberg, Germany, IRMS = IsoPrime by Micromass UK Limited, Manchester, UK). For analyses of P-concentration in soil, 50 mg material were dissolved for 30 minutes with 0.25 g of a lithium-boron-mixture at 1000°C than solubilized in 30 ml 5% HNO_3 and filled to a volume of 50 ml with deionized water. P-concentration [%] was determined with ICP-OES (inductively coupled plasma with optical emission spectroscopy, Thermo Fisher Scientific, Waltham, USA). N_{min} [ppm] was analyzed in the soil solution of 5 g fresh soil, shaken with 50 ml of 1 M KCl for 0.5 hours. Determination of ammonium (NH_4^+) and nitrate (NO_3^-); concentration was done with an IC system (ion chromatography: Dionex ICS 3000 SP with ICS 3000 DC conductivity detector, AD 25 UV-VIS detector, by Dionex Corporation, Sunnyvale, USA).

$\delta^{15}\text{N}$ -methods

We used the $\delta^{15}\text{N}$ natural abundance method (Amarger et al., 1979) to study positive effects of N_2 fixed by legumes on neighboring plants, which was adapted from the method of Shearer & Kohl (1986) for estimating percent N derived from atmosphere (%Ndfa) in N_2 -fixing species. The $\delta^{15}\text{N}$ natural abundance signal denotes the ratio of the heavier over the lighter stable isotope of nitrogen (^{15}N over ^{14}N) in a sample in relation to a standard (atmospheric N_2 for nitrogen as described by Mariotti (1983)):

$$\delta^{15}\text{N} = [(R_{\text{sample}}/R_{\text{standard}}) - 1] * 1000 [\text{‰}]. \quad (1)$$

Where R_{sample} or R_{standard} is the ratio of ^{15}N over ^{14}N for sample or standard, respectively.

To achieve a higher comparability between plant species growing in different soils, we also standardized plant $\delta^{15}\text{N}$ values with the bulk soil signal as recommended by Amundson et al. (2003) and Kahmen et al. (2008). We subtracted the $\delta^{15}\text{N}$ value of the bulk soil ($\delta^{15}\text{N}_{\text{soil}}$) from the plant signal ($\delta^{15}\text{N}_{\text{foliar}}$) to obtain a so-called big delta signal ($\Delta\delta^{15}\text{N}$):

$$\Delta\delta^{15}\text{N} = \delta^{15}\text{N}_{\text{foliar}} - \delta^{15}\text{N}_{\text{soil}} [\text{‰}]. \quad (2)$$

We estimated percentage of plant nitrogen derived from atmosphere (%Ndfa) in the legume species according to Shearer & Kohl (1986):

$$\% \text{Ndfa} = ((\delta^{15}\text{N}_{\text{ref}} - \delta^{15}\text{N}_{\text{fix}})/(\delta^{15}\text{N}_{\text{ref}} - B)) * 100 [\%]. \quad (3)$$

Where $\delta^{15}\text{N}_{\text{ref}}$ and $\delta^{15}\text{N}_{\text{fix}}$ are the stable isotope ratios measured in the non-fixing reference (ref) and N_2 -fixing species (fix). B refers to the $\delta^{15}\text{N}$ signal of the nodulated N_2 -fixing species growing in a media totally lacking in mineral N and thus solely dependent on N from atmosphere. Instead of using a legume, which solely depends on Ndfa, to gain the B value, we used the lowest value of the field-grown legume species as B (as recommended by

Eriksen & Høgh-Jensen (1998)). To use %Ndfa-method, the $\delta^{15}\text{N}_{\text{ref}}$ should be higher (more positive) than the $\delta^{15}\text{N}_{\text{fix}}$ ($\delta^{15}\text{N}_{\text{ref}} > \delta^{15}\text{N}_{\text{fix}}$), thus we used the mean value from *G. mollugo* from the -r-h areas as the $\delta^{15}\text{N}_{\text{ref}}$ species to gain a rough estimate for %Ndfa for all sampled legume species in all restoration treatments (Table 3).

Mycorrhizal fungi-staining

For randomly chosen roots from every transect, we did a trypan blue root staining (Phillips & Hayman, 1970) to assess if an infection with mycorrhizal fungi (MF) had occurred. We checked for MF hyphae in- and outside the root cortex and for the formation of vesicles and arbuscules inside the tissue by microscopic observation but did not determine percentage of infected root length, as we did not know which species the roots belonged to.

Table 3 Estimation of the percent of nitrogen derived from atmosphere (%Nd_{fa}) from N₂-fixation in four legume species in four areas/restoration treatments with (a) results for percent N derived from atmosphere (%Nd_{fa}) for the legume species and (b) B values ($\delta^{15}\text{N}$ value [‰]) of a legume that depends predominantly on N₂-fixation for its N source, see eq. 3) which was the lowest $\delta^{15}\text{N}$ values of each field-grown legume species in our study (*sensu* Eriksen & Høgh-Jensen (1998)). *Galium mollugo* from control areas (-r-h, $\delta^{15}\text{N} = -0.94$ ‰) was used as a reference plant ($\delta^{15}\text{N}_{\text{ref}}$) for all calculations.

(a) %Nd _{fa}	+r+h	+r-h	-r+h	-r-h
ant	76.38	73.31	75.77	85.72
dor	155.01	85.37	n.a.	n.a.
lot	n.a.	71.98	63.16	73.39
tri	n.a.	n.a.	n.a.	63.45
(b) B value [‰]				
ant	-2.80	-2.90	-2.60	-2.80
dor	-2.66	-2.70	n.a.	n.a.
lot	n.a.	-2.80	-3.00	-3.00
tri	n.a.	n.a.	n.a.	-1.90

Restoration treatments: with (+) or without (-) topsoil removal (r) and hay transfer (h)

Target legume species: ant = *Anthyllis vulneraria*, dor = *Dorycnium germanicum* and mesic legume species: lot = *Lotus corniculatus*, tri = *Trifolium pratense*

n.a. = species not available in this treatment

Statistical analysis

The restoration project has a two factor, full-factorial design: an abiotic factor, topsoil removal (r) with two levels (removal and no removal), and a biotic factor, hay transfer (h) with two levels (hay transfer and no hay transfer), giving four restoration treatments (+r+h, +r-h, -r+h and -r-h). The block effect was negligible for most subsets of data. We used all data together only for one analysis (for all species over all restoration treatments), all other analyses were performed using subsets of data separated either by functional identity (FI), species identity (SI), neighborhood (NH) or restoration treatment.

Before statistical analyses, all data were tested for homogeneity of variance (Levene's test) and normality (Kolmogorov-Smirnov test, Q-Q-Plots), and log transformed if assumptions were not met. An overall ANOVA (Type III Sum of - Table 4) was performed with topsoil removal (r), and hay transfer (h) as fixed factors. We performed one ANOVA for the whole dataset (all species in all restoration treatments together, n of all samples = 293, Table 4a), two separate ANOVA for effects of topsoil removal and hay transfer on legume species (n = 126) and non-legume species (n = 167), respectively (Table 4b) and one separate ANOVA for each species (*A. vulneraria*: n = 46, *D. germanicum*: n = 18, *H. nummularium*: n = 85, *L. corniculatus*: n = 47, *T. pratense*: n = 15, *G. mollugo*: n = 82; Table 4c).

Four separate ANOVA (sequential Type I Sum of Squares - to test effects of single factors which were interrelated with each other) were performed to test (*within* a single restoration

treatment) for effects of functional (FI) or species identity (SI) as well as the neighborhood effect (NH; only for non-legume species). We used FI, SI and NH as fixed factors in the analyses for each of the four restoration treatments (-r-h: n = 79, -r+h: n = 59, +r-h: n = 82, +r+h: n = 73; Table 5). We used least-significant-difference test (LSD) as a post-hoc test when significant differences were found between treatments.

Soil and root data (unlike the single plant data), were pooled per transect (one transect of the same restoration treatment per block, giving n = 2). When significant differences between treatments were found, we used LSD post-hoc tests to distinguish where the differences were.

RESULTS

Restoration treatment effects on soil

Hay transfer had no effect on soil nutrient parameters (except for $\text{NH}_4^+:\text{NO}_3^-$ -ratios: $p = 0.014$) but topsoil removal was very effective in reducing plant available nutrients (Table 1a, Fig. 1): topsoil removal reduced both mineral N-forms (N_{min} : NH_4^+ and NO_3^-), total N- and P-concentrations strongly by 60-70%, respectively ($p \leq 0.020$). The $\text{N}_{\text{min}}:\text{N}$ [%]-ratios were relatively stable over the different treatments ($p = 0.162$; Fig. 1) but the ratio of ammonium to nitrate ($\text{NH}_4^+:\text{NO}_3^-$) was significantly higher in topsoil removal areas than in non-removal areas ($p < 0.001$) and this corresponded to extremely low nitrate concentrations after topsoil removal (Fig. 1). Topsoil removal reduced $\delta^{15}\text{N}$ values in bulk soil on average by 1.28 ‰ compared to non-removal areas (+r: 3.23 ‰ < -r: 4.51 ‰, $p = 0.005$).

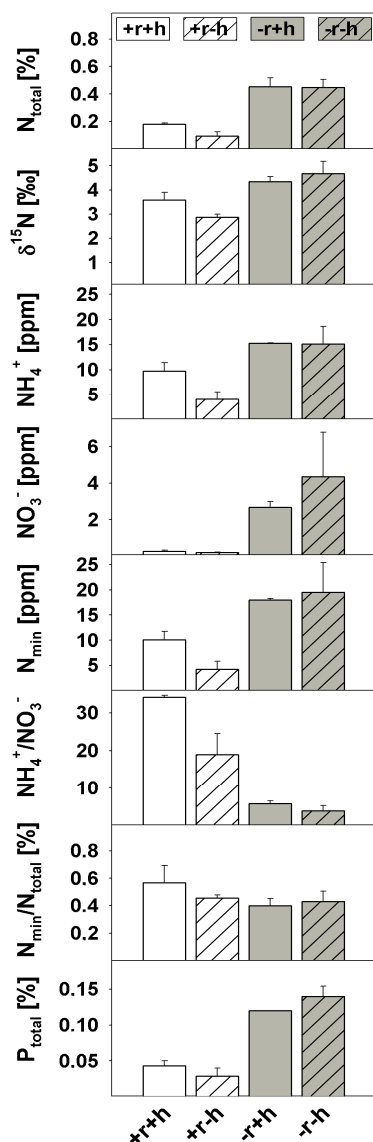


Figure 1 Soil properties of the four restoration treatments in 2008 (see also Table 1a); parameters include N_{total} and P_{total} (concentrations [%] measured in bulk soil; fraction <2 mm) and mineral N-forms (ammonium (NH_4^+), nitrate (NO_3^-) and both together (N_{min}) measured in soil solution). Values are means \pm 1 standard deviation. Restoration treatments are: with topsoil removal and hay transfer (+r+h, white bars), with topsoil removal and without hay transfer (+r-h, white striped bars), without topsoil removal and with hay transfer (-r+h, gray bars), without topsoil removal and without hay transfer (-r-h, gray striped bars).

Restoration treatment effects on roots

All unspecific root samples contained some nodulated legume roots. Root samples did not vary in their N-concentration between restoration treatments ($p = 0.368$; Fig. 2) but the $\delta^{15}\text{N}_{\text{root}}$ values were significantly lower ($p = 0.016$) in topsoil removal areas than in non-removal areas. After standardizing $\delta^{15}\text{N}_{\text{root}}$ against bulk soil $\delta^{15}\text{N}_{\text{soil}}$ ($\Delta\delta^{15}\text{N}_{\text{root}}$, right-hand column in Fig. 2), this significant difference disappeared ($p = 0.069$). All root samples were heavily infected with mycorrhizal fungi (50 - 95% of the root tissue per sample, data not shown). We found vesicles, arbuscules, internal and external hyphae, which are typical structures denoting vesicular-arbuscular mycorrhiza (VAM). Other kinds of fungal material were also visible; some of the significantly thicker, brownish stained material may have been ectomycorrhizal fungi components which often occur in symbiosis with *Helianthemum nummularium* (Harley & Harley, 1987).

Restoration treatment effects on leaves

Restoration treatments affected N-concentration as well as $\delta^{15}\text{N}$ and $\Delta\delta^{15}\text{N}$ values very significantly in leaf tissues of the six plant species sampled (Table 4a, Figs. 2 & 3). A significant interaction of topsoil removal and hay transfer (r^*h) was detected for N-concentration and for $\Delta\delta^{15}\text{N}$ (standardized with $\delta^{15}\text{N}_{\text{soil}}$) but not for $\delta^{15}\text{N}$ values (Table 4a).

When data were split into subsets based on functional identity (FI; legume or non-legume species) and then tested for effects of restoration treatments, we also found very significant effects of topsoil removal and hay transfer on N-dynamics (Table 4b), but no interaction (r^*h).

When data were split into subsets based on species identity (SI), topsoil removal and hay transfer affected N-parameters of most species significantly (Table 4c, Fig. 2). N-concentration increased with decreasing restoration effort (\triangleq decreasing environmental severity; from topsoil removal with hay transfer areas (+r+h) to non-removal and no hay transfer areas (-r-h)) for *Anthyllis vulneraria*, *H. nummularium* and even stronger for the mesic forb *Galium mollugo*. In both non-legume species (*H. nummularium*, *G. mollugo*) the increase in N-concentration was accompanied by an increase in $\delta^{15}\text{N}_{\text{foliar}}$ (values get less negative, Fig. 2). Changes in $\delta^{15}\text{N}_{\text{non-legumes}}$ were even more pronounced than changes in N-concentration and

reflected restoration effort very well. The difference in $\delta^{15}\text{N}$ and N-concentration values between +r and -r areas were bigger for *G. mollugo* than for *H. nummularium*.

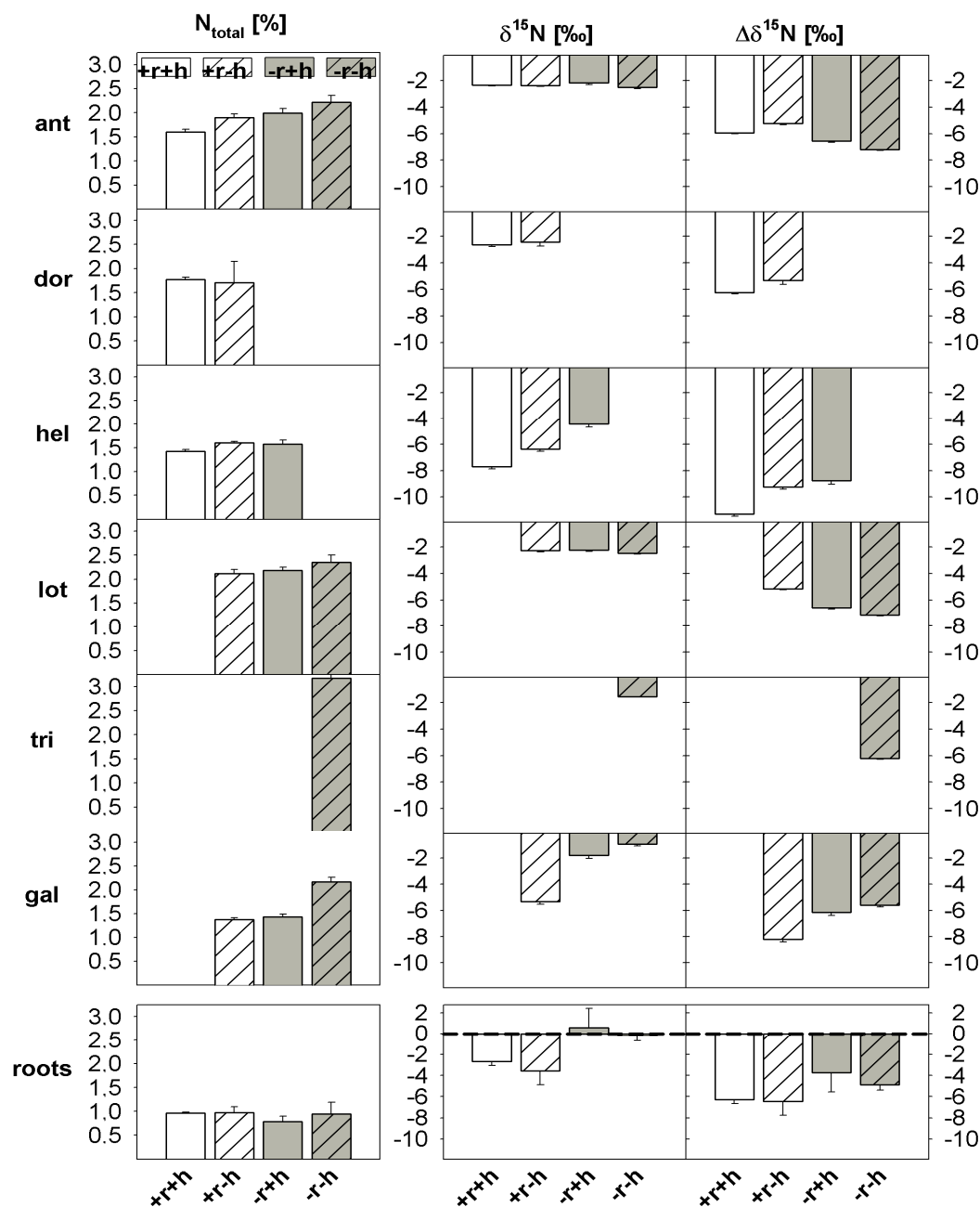


Figure 2 N-concentration [%], $\delta^{15}\text{N}$ [‰] and $\Delta\delta^{15}\text{N}$ [‰] values in plant leaves of all six species (three target calcareous grassland and three mesic species) and in root samples (species not identified) in the four restoration treatments. Values are means \pm 1 standard deviation (plant: $n = 6 - 42$, except for *D. germanicum* in +r-h treatment: $n = 2$; root: $n = 2$). Data (and thus bars) for some species in some treatments are missing, as the species was not growing in that treatment (see Table 2). Restoration treatments are: with topsoil removal and hay transfer (+r+h, white bars), with topsoil removal and without hay transfer (+r-h, white striped bars), without topsoil removal and with hay transfer (-r+h, gray bars), without topsoil removal and without hay transfer (-r-h, gray striped bars). Target species: ant = *Anthyllis vulneraria*, dor = *Dorycnium germanicum*, hel = *Helianthemum nummularium* (two legume and one non-legume species, respectively) and mesic species: lot = *Lotus corniculatus*, tri = *Trifolium pratense* and gal = *Galium mollugo* (two legume and one non-legume species, respectively).

Table 4 ANOVA results (Type III Sum of Squares) for effects of topsoil removal (r), hay transfer (h) and their interaction effect (r*h) on N-parameters (N-concentration [%], $\delta^{15}\text{N}$ and $\Delta\delta^{15}\text{N}$ [‰]) in plant leaves for (a) all data analyzed together, (b) data split into functional identities (legumes versus non-legumes) and then tested for restoration treatment effects and (c) data analyzed per species and tested for effects of restoration treatments.

Factor		d.f.	N [%]		$\delta^{15}\text{N}$		$\Delta\delta^{15}\text{N}$	
			<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
(a) All species								
	r	1	65.025	< 0.001	194.035	< 0.001	66.938	< 0.001
	h	1	61.378	< 0.001	17.477	< 0.001	25.295	< 0.001
	r*h	1	15.664	< 0.001	0.018	0.892	6.105	0.014
(b) Functional identity								
non-	r	1	34.737	< 0.001	783.733	< 0.001	443.693	< 0.001
leg.	h	1	57.936	< 0.001	77.090	< 0.001	96.111	< 0.001
	r*h	1	25.901	< 0.001	0.110	0.740	9.907	0.002
leg.	r	1	57.228	< 0.001	12.082	0.001	180.230	< 0.001
	h	1	29.693	< 0.001	4.028	0.047	20.275	< 0.001
	r*h	1	0.631	0.428	0.113	0.737	51.863	< 0.001
(c) Species identity								
ant	r	1	16.379	< 0.001	0.001	0.976	172.441	< 0.001
	h	1	8.646	0.005	3.320	0.076	0.015	0.904
	r*h	1	0.434	0.514	2.746	0.105	49.048	< 0.001
dor	r	0						
	h	1	0.391	0.541	0.383	0.544	7.133	0.017
	r*h	0						
hel	r	1	4.501	0.037	74.492	< 0.001	44.033	< 0.001
	h	1	15.994	< 0.001	32.447	< 0.001	74.982	< 0.001
	r*h	0						
lot	r	1	2.192	0.146	1.944	0.170	250.837	< 0.001
	h	1	0.800	0.376	2.974	0.092	19.570	< 0.001
	r*h	0						
tri	only in -r-h	n.a.						
gal	r	1	40.278	< 0.001	203.595	< 0.001	70.726	< 0.001
	h	1	49.685	< 0.001	11.124	0.001	4.214	0.043
	r*h	0						

Target species: ant = *Anthyllis vulneraria*, dor = *Dorycnium germanicum*, hel = *Helianthemum nummularium* and mesic species: lot = *Lotus corniculatus*, tri = *Trifolium pratense* (not tested because it only occurred in one treatment), gal = *Galium mollugo*

Standardization of $\delta^{15}\text{N}_{\text{foliar}}$ with $\delta^{15}\text{N}_{\text{soil}}$ ($\Delta\delta^{15}\text{N}_{\text{foliar}}$) for single species (Fig. 2, right-hand column) revealed a pronounced increase in $\Delta\delta^{15}\text{N}$ (values got less negative) with decreasing restoration effort for the non-legume species whereas the $\Delta\delta^{15}\text{N}$ value decreased (got more negative) for all legume species. Mean $\Delta\delta^{15}\text{N}$ values were in the same range for all herbaceous species (-5 to -7.5 ‰) whereas *H. nummularium*, a small shrub, showed higher deviations from $\delta^{15}\text{N}_{\text{soil}}$.

When data were tested for effects of functional identity (FI) or species identity (SI) *within* each restoration treatment (data split into four subsets; see Table 5) both FI and SI affected all foliar N-parameters. Effects were stronger in the topsoil removal areas than in non-removal areas.

Table 5: ANOVA (sequential Type I) results for effects of functional (FI) and species identity (SI) as well as effect of neighborhood (NH; legume-neighbor or not for non-legume species) on N-parameters for each restoration treatment separately (with (+) or without (-) topsoil removal (r) and hay transfer (h)).

		N [%]		$\delta^{15}\text{N}$		$\Delta\delta^{15}\text{N}$		
Factor	d.f.	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	
-r-h	FI	1	14.944	< 0.001	52.241	< 0.001	52.241	< 0.001
	SI	2	8.169	0.001	7.556	0.001	7.556	0.001
	NH	2	0.200	0.819	1.416	0.249	1.416	0.249
-r+h	FI	1	80.073	< 0.001	1.257	0.267	1.257	0.267
	SI	2	2.542	0.088	27.872	< 0.001	27.872	< 0.001
	NH	1	5.755	0.020	0.484	0.490	0.484	0.490
+r-h	FI	1	79.097	< 0.001	465.050	< 0.001	465.050	< 0.001
	SI	3	8.431	< 0.001	6.559	0.001	6.559	0.001
	NH	4	4.745	0.002	2.334	0.064	2.334	0.064
+r+h	FI	1	37.154	< 0.001	687.014	< 0.001	687.014	< 0.001
	SI	1	5.650	0.020	0.954	0.332	0.954	0.332
	NH	2	8.225	0.001	2.497	0.090	2.497	0.090

Nitrogen-fixation in legumes and N-facilitation along an environmental stress gradient

The legume species derived on average approximately 82% of their N from atmosphere (Nd_{fa}, Table 3), which corresponds well with their constant $\delta^{15}\text{N}$ values in all restoration treatments (around -2‰, Fig. 2 & 3). These values indicate high levels of N₂-fixation with low amounts of N derived from the soil, thus the legume species studied would be able to provide a source of atmospherically fixed N for non-legume neighbors. In our study, $\delta^{15}\text{N}$ values in both non-legume species growing in topsoil removal treatments were much more negative than the $\delta^{15}\text{N}$ signals of the legumes.

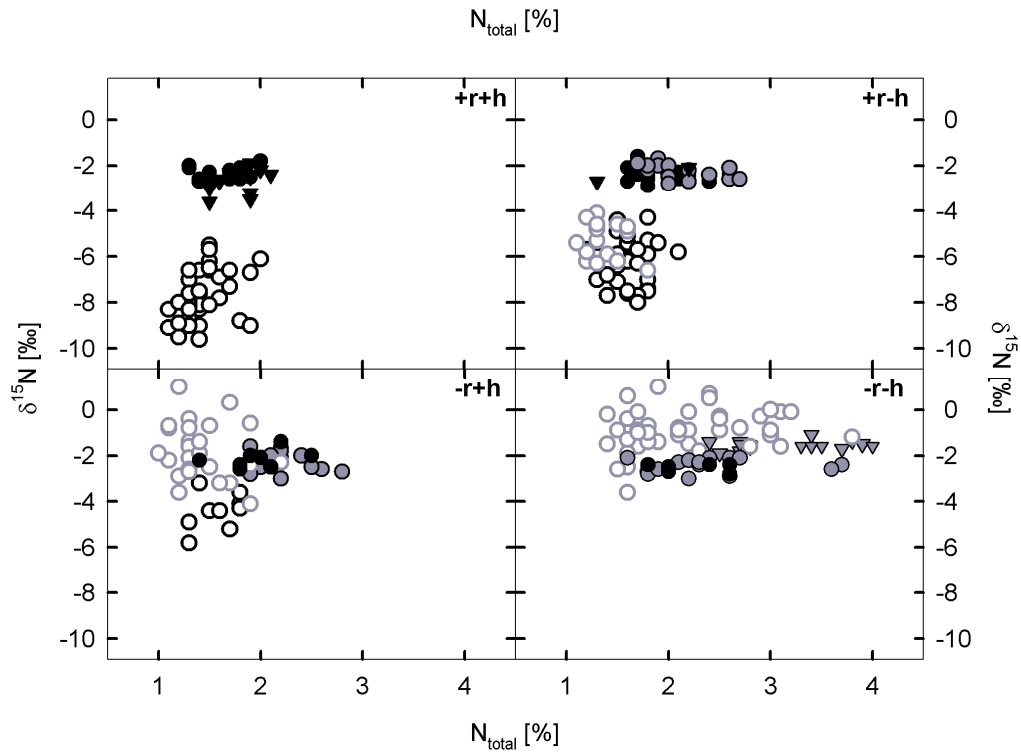


Figure 3 The relationship between $\delta^{15}\text{N}$ values and N-concentration [%] in leaves of the six plant species separated into panels for each restoration treatment. Restoration treatments are: with topsoil removal and hay transfer (+r+h), with topsoil removal and without hay transfer (+r-h), without topsoil removal and with hay transfer (-r+h), without topsoil removal and without hay transfer (-r-h). Legume species have closed symbols, non-legume species have open symbols; target species have black symbols: *Anthyllis vulneraria* (●) and *Dorycnium germanicum* (▼), *Helianthemum nummularium* (black-open: ○) and mesic, non-target species have gray symbols: *Lotus corniculatus* (●), *Trifolium pratense* (▼) and *Galium mollugo* (gray-open: ○). Every symbol represents one replicate per species and restoration treatment (for mean values per species see Fig. 2). Neighborhood effects of legumes on non-legume species are shown in Figure 4.

The non-legume control plants seldom differed from the legume-neighbor plants in $\delta^{15}\text{N}$ and a little more frequently in N-concentration values but never in both N-parameters simultaneously (Table 5, Fig. 4). The N-concentration was higher in control plants if significant differences occurred. It was possible to compare legume neighbor effects on *G. mollugo* (but not on *H. nummularium*) between topsoil removal and non-removal areas. In control areas (-r-h) no differences occurred between *G. mollugo* as control plant or as neighbor of *L. corniculatus* whereas in topsoil removal areas (+r-h) *G. mollugo* as neighbor of *L. corniculatus* had higher $\delta^{15}\text{N}$ values (closer to that of the legume species) than control plants (Fig. 4). The N-concentration was not affected by the legume neighbor.

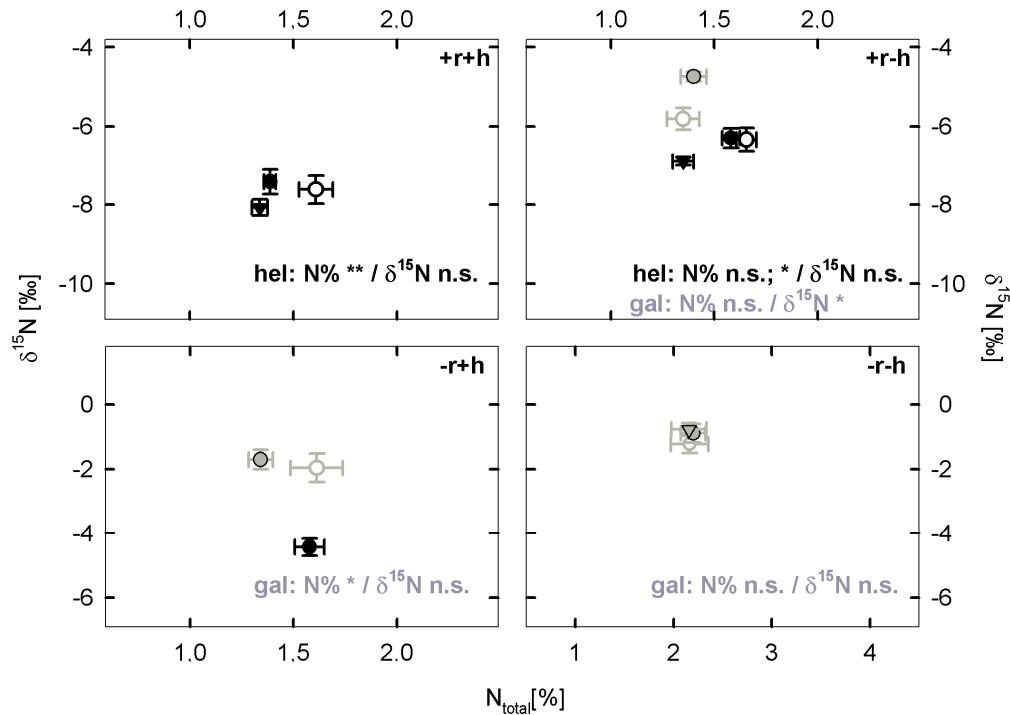


Figure 4 The relationship between $\delta^{15}\text{N}$ and N-concentration [%] in leaves of the two non-legume species (hel: *Helianthemum nummularium* and gal: *Galium mollugo*) as affected by the presence of two different legume species in the neighborhood. The restoration treatment panels are the same as in Figure 4; values are means with standard error of the mean. *H. nummularium* (black type and symbols) was collected as a neighbor of the legumes *Anthyllis vulneraria* (●) or *Dorycnium germanicum* (▼) and as a control (○) without legume neighbors. Similarly, *G. mollugo* (gray type and symbols) was collected as a neighbor of the legumes *Lotus corniculatus* (●) or *Trifolium pratense* (▼) and as control (○) without legume neighbors. The number of samples per species (see Table 2) varied with species and treatment but in general $n = 8 - 16$, (only *H. nummularium* as neighbor of *D. germanicum* in +r-h had $n = 2$). Significant effects of legume neighborhood on $\delta^{15}\text{N}$ and N-concentration within each restoration treatment are shown as $p < 0.05 = *$, $p < 0.001 = **$, n.s. = not significant, p -values from LSD-tests.

Relationship between environmental factors and foliar ^{15}N -parameters

There were strong relationships between ^{15}N -parameters in leaves of non-legume species ($\delta^{15}\text{N}_{\text{non-legumes}}$ and $\Delta\delta^{15}\text{N}_{\text{non-legumes}}$) and biotic (see Table 1b) and abiotic (see Fig. 1) parameters from the restoration sites (Fig. 5). Mean values of ^{15}N -parameters for *G. mollugo* and *H. nummularium* showed a close link to target species richness (richness of species adapted to calcareous grasslands) but not with total species richness per restoration treatment. Also, ^{15}N -parameters showed close links to soil $\text{NH}_4^+:\text{NO}_3^-$ -ratios whereas the $\delta^{15}\text{N}$ signal of the soil was well reflected only in ^{15}N -parameters of *G. mollugo* but not in those of *H. nummularium*. Standardization of $\delta^{15}\text{N}_{\text{foliar}}$ with $\delta^{15}\text{N}_{\text{soil}}$ ($\Delta\delta^{15}\text{N}$) did not ameliorate the differences in $\delta^{15}\text{N}$ values of non-legume species along the environmental nutrient gradient.

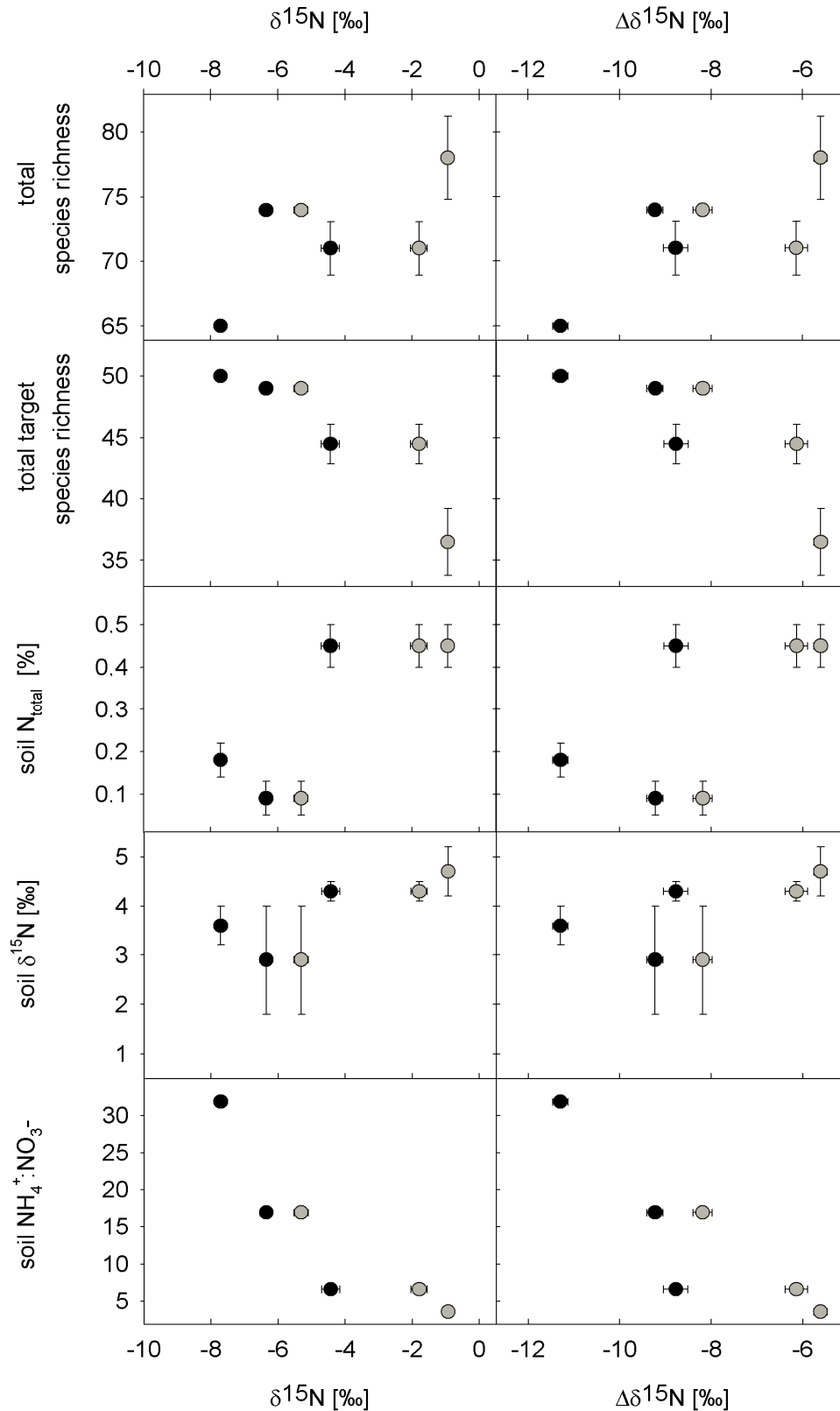


Figure 5 Relationship between foliar $\delta^{15}\text{N}$ (left-hand column) and $\Delta\delta^{15}\text{N}$ ($\delta^{15}\text{N}_{\text{foliar}}$ minus $\delta^{15}\text{N}_{\text{soil}}$; right-hand column) values and biotic and abiotic parameters of the restoration areas. Biotic parameters include total species richness and total target species richness (adapted to calcareous grasslands) per restoration treatment; abiotic parameters (bulk soil) include N-concentration, $\delta^{15}\text{N}_{\text{soil}}$ values and $\text{NH}_4^+:\text{NO}_3^-$ -ratios for all four restoration treatments. Values are means ± 1 standard deviation, black symbols (●) represent *H. nummularium*, gray symbols (●) *G. mollugo*; each symbol is for the foliar $\delta^{15}\text{N}$ and $\Delta\delta^{15}\text{N}$ value of a species (without separation in different neighborhoods) in a given biotic or abiotic environment in one restoration treatment.

DISCUSSION

Applicability of the $\delta^{15}\text{N}$ natural abundance method to study N-facilitation in nutrient-poor grasslands

Although the $\delta^{15}\text{N}$ natural abundance method has been successfully used to assess facilitation between legumes and their neighbors in mesic temperate experimental grasslands (Mulder et al., 2002; Temperton et al., 2007), it has been barely tested in nutrient-poor grasslands, such as calcareous grasslands in Central Europe.

Facilitation studies that have successfully used the $\delta^{15}\text{N}$ method generally also had legume-free control communities, but such conditions are very hard to find in semi-natural grasslands. However, Jacot et al. (2005) found that neighboring legume species generally affected the $\delta^{15}\text{N}$ signals of non-legume neighbors in different semi-natural grasslands in the Alps. Bai et al. (2009) found clear convergence of $\delta^{15}\text{N}$ signals of non-legume shrubs towards that of neighboring N_2 -fixing species with decreasing distance between non-fixing and fixing species in natural subtropical savannah communities.

All these studies have in common, that the $\delta^{15}\text{N}$ values of non-legume species are higher than those of their legume neighbors, whereas in our study, we generally found that $\delta^{15}\text{N}$ signals in the non-legumes were much more depleted in ^{15}N (had more negative values) than in the legumes. Decreases in foliar N-parameters are often related to decreasing N-availability in the substrate, lower nitrification and mineralization rates and thus an overall more closed N-cycle (e.g. Pardo et al., 2006; Kahmen et al., 2008). Interestingly, $\delta^{15}\text{N}$ values of the non-fixing target species at our calcareous grassland restoration site corresponded well with values found for non-legume species in other (acidic) low-nutrient ecosystems: *Helianthemum nummularium* ranged from -9.6 to -3.2‰, which corresponds well with negative $\delta^{15}\text{N}$ values found in Dutch sand dunes (van der Heijden et al., 2006).

Beyschlag et al. (2009) studied N-facilitation in two different successional states (as surrogates for environmental severity) of an acidic dry grassland and found no clear positive legume effects on neighboring non-legume species (with negative $\delta^{15}\text{N}$ values), either in natural communities or in an additional legume removal experiment (although data showed a trend for positive legume effects on biomass and $\delta^{15}\text{N}$ values). The $\delta^{15}\text{N}$ method (although not explicitly so-stated by Shearer & Kohl (1986) or Robinson (2001)), may require that the $\delta^{15}\text{N}$ signal of the non-legume neighbor be more positive than that of the N_2 -fixing legume (with

control plants having the most positive ^{15}N signals). Our work indicates that further work on N-facilitation by legumes is needed to assess, under what range of soil nutrient conditions the $\delta^{15}\text{N}$ natural abundance method can be effectively used to study this kind of plant-plant interaction in N-limited systems.

Testing the stress gradient hypothesis: Facilitation along an environmental stress gradient as provided by four different restoration treatments

In accordance with Schulze et al. (1994), who found that differences in $\delta^{15}\text{N}$ values between different plant life forms disappear with increasing N-availability in the substrate, we found a gradual convergence of $\delta^{15}\text{N}$ signals in non-legume and legume species when moving from the most restoration intensive sites (topsoil removal and hay transfer, +r+h) to control sites (-r-h, Fig. 3). Decreasing $\delta^{15}\text{N}$ values with increasing environmental severity have also been found in grasslands in the Alps where the $\delta^{15}\text{N}$ value in plants decreased with increasing altitude (Huber et al. (2007) and Jacot et al. (2005)). Jacot et al. (2005) found an increase in the difference between $\delta^{15}\text{N}$ values of legume species and non-legume species with increasing altitude and thus evidence for the applicability of the $\delta^{15}\text{N}$ natural abundance method to test the stress gradient hypothesis in this habitat (Bertness & Callaway, 1994). In our lowland calcareous grassland restoration site and in a dry acidic grassland (Beyschlag et al. 2009), however, abiotic conditions, as well as species' identity, seem to have had a much stronger effect on N-characteristics of the plant species than the biotic interactions with neighboring legumes.

It was not possible to clearly assess the strength of facilitative interactions at our site maybe due to very depleted (negative) $\delta^{15}\text{N}$ values in non-legume species but also due to the general lack of control non-legume plants at a large enough distance (>1 m) from a legume species at this restoration site. For this reason, the only clear evidence for legume facilitation was found in *G. mollugo*, which had an increased amount of leaf nitrogen originating from neighboring *Lotus corniculatus* (identified using the $\delta^{15}\text{N}$ signal) when N-limitation in the substrate increased (areas without hay transfer in Fig. 4). There was no clear evidence for N derived from legume neighbors, however, in the other non-legume species *H. nummularium*, which possibly profited more from N-sources provided by mycorrhizal fungi (see below for discussion).

Impact of edaphic and biotic factors on $\delta^{15}\text{N}_{\text{foliar}}$

Soil N-availability, mycorrhizal status, plant species identity and the surrounding species richness, N-source as well as the life-form of the species under investigation strongly affect N-concentration and $\delta^{15}\text{N}$ signal in non-fixing plant species (e.g. Schulze et al., 1994; Högberg, 1997; Pardo et al., 2006; Temperton et al., 2007). These factors seem to have been more important for determining the $\delta^{15}\text{N}$ signal of non-legume plants than effects of neighboring legume species in our calcareous grassland site. Differentiating mechanistically between the exact role of different factors in affecting the $\delta^{15}\text{N}$ signal of plants is usually not possible in natural communities, and requires studies either under controlled conditions (e.g. Paynel & Cliquet, 2003) or with enriched stable isotope tracers in the field (Hogh-Jensen, 2006; Gylfadottir et al., 2007; Kahmen et al., 2008). A number of studies using the $\delta^{15}\text{N}$ natural abundance method (alone or combined with enriched tracers), however, provide some key pointers which help to interpret how our results ($\delta^{15}\text{N}$ signals on calcareous grassland restoration sites) correlated with soil nutrient status.

Low foliar $\delta^{15}\text{N}$ values generally correspond to low N-availability (e.g. Pardo et al., 2006; Kahmen et al., 2008). Our results confirm this general relationship: we found lowest mean $\delta^{15}\text{N}$ values in *H. nummularium* (-7.7 ‰) and *G. mollugo* (-5.3 ‰, Fig. 2 & 3) in topsoil removal areas and significantly higher values in non-removal areas. The most depleted $\delta^{15}\text{N}$ values were in the same range as in nutrient-poor acidic grasslands on sea or inland dunes (van der Heijden et al., 2006; Beyschlag et al., 2009) but significantly lower than results reported from mesic (natural and experimental) grassland systems which normally report positive $\delta^{15}\text{N}$ values (+2 to +6 ‰) for non-legume herbaceous species (Mulder et al., 2002; Spehn et al., 2002; Temperton et al., 2007; Kahmen et al., 2008). Kahmen et al. (2008) investigated plant $\Delta\delta^{15}\text{N}$ (i.e. plant values standardized by soil ^{15}N background values) in relation to N-uptake preferences from the soil and found decreasing $\Delta\delta^{15}\text{N}_{\text{foliar}}$ values with increasing proportion of NH_4^+ uptake (i.e. high $\text{NH}_4^+:\text{NO}_3^-$ -ratios). Our results confirmed this trend for the two non-legume species studied, and in our case relationships between $\text{NH}_4^+:\text{NO}_3^-$ and foliar $\delta^{15}\text{N}$ values were even stronger than with the standardized $\Delta\delta^{15}\text{N}$ values in both species (Fig. 5)). The opposite relationship, however, was found by Miller and Bowman (2002) - probably because the analysis of soil N_{min} provides only a snapshot of soil N-dynamics such that correlations between $\delta^{15}\text{N}_{\text{foliar}}$ and soil N_{min} need testing over longer periods of the growing season.

Decreasing $\delta^{15}\text{N}_{\text{foliar}}$ in *G. mollugo* reflected increasing $\text{NH}_4^+:\text{NO}_3^-$ -ratios in the soil at the restoration sites (and to a lesser extent in *H. nummularium*, too; Fig. 5), which suggests that these species either preferably took up larger proportions of severely ^{15}N -depleted ammonium or that, with a decreasing amount of nitrate in the substrate (Fig. 1), other N-acquisition strategies (e.g. via mycorrhizal symbiosis) became more important. Weak links between abiotic/biotic parameters and ^{15}N -parameters (Fig. 5) as well as lower $\Delta\delta^{15}\text{N}$ values (Fig. 2) in *H. nummularium* than in all other species suggest effects of other factors additional to the impact of the abiotic soil environment. We hypothesize that the differences found between the two non-legume species may be attributable to their different mycorrhizal symbioses and life-histories. *H. nummularium* has two features normally connected to low $\delta^{15}\text{N}$ values: (a) it is a perennial shrub with woody parts and (b) forms a symbiosis with ectomycorrhizal fungi (ECM); whereas the forb *Galium mollugo* is associated with arbuscular mycorrhiza fungi (AM) (Harley & Harley, 1987). It is known that $\delta^{15}\text{N}$ values of non-fixing plants decline with longevity and woodiness of the species (Virginia & Delwiche, 1982), and that ECM normally decreases $\delta^{15}\text{N}_{\text{foliar}}$ values more strongly than AM (Michelsen et al., 1998; Spriggs et al., 2003).

Conclusions: The potential for using $\delta^{15}\text{N}$ signals in plants as indicators of restoration success in nutrient-poor grassland systems

Both ^{15}N -parameters ($\delta^{15}\text{N}$ and $\Delta\delta^{15}\text{N}$ values) in leaves of calcareous grassland species at our restoration site did not provide clear information on the strength of facilitation but rather seemed to provide important integrative information about the N-dynamics in the soil (as well as potential effects of mycorrhizal symbioses). The most depleted values were found in the topsoil removal treatments and the most enriched values in the control treatments without soil removal and hay transfer, thus $\delta^{15}\text{N}$ values became more depleted with increasing environmental severity and higher restoration effort. Other studies in a range of habitats have also shown that $\delta^{15}\text{N}$ in plants can be a useful overall integrator of changing N-dynamics in the soil, and in our restoration experiment it seems that the ^{15}N signal derived from soil N-dynamics was much stronger than that for N-facilitation from legume neighbors. This finding can be useful for the assessment of restoration success in formerly eutrophic habitats in need of nutrient reduction (with high/positive $\delta^{15}\text{N}$ values) to restore them to high-diversity, low nutrient systems (with low/negative $\delta^{15}\text{N}$ values). Thus using $\delta^{15}\text{N}$ signals of plants that

integrate N-dynamics over time in combination with traditional soil nutrient analyses could provide a relatively simple indicator of N-dynamics of the system. The congruence between target species richness and $\delta^{15}\text{N}$ values in non-legume species corroborate this conclusion.

High atmospheric N deposition and overloading of soils with N and P fertilisers are common problems related to degradation and species loss in Central and Northern European habitats (Verhagen et al., 2001; Walker et al., 2004; Verhagen & van Diggelen, 2006). As such, analyzing $\delta^{15}\text{N}$ in plants (in combination with assessing vegetation changes after restoration), could provide a relatively simple tool to assess restoration success (reducing soil N) in systems that are stuck in an undesirable alternative stable state (Hobbs & Norton, 2004).

“IMPLICATIONS FOR PRACTICE”-BOX

- Increasing restoration effort (and thus environmental severity) in this calcareous grassland restoration project resulted in increased calcareous grassland species richness, which corresponded to decreasing $\delta^{15}\text{N}$ values of different non-legume species.
- Negative foliar $\delta^{15}\text{N}$ values of non-legume species provided evidence for increasing N-limitation with increasing restoration effort in our study and hence can be used for the evaluation of restoration success during the creation of low-productive grasslands. Generally, higher N-limitation is related to a more closed N-cycle which corresponds to more negative $\delta^{15}\text{N}$ values.
- Foliar $\delta^{15}\text{N}$ values of non-legume species (in combination with vegetation relevés) could be used as an indicator of relative restoration success: foliar $\delta^{15}\text{N}$ values in such calcareous grasslands could provide integrated information about the N-status of the soil, and hence, in this case, restoration success (when restoring a system back from a “high N with medium diversity system” to a “low N with high diversity system”).

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MANUSCRIPT 4

N-TRANSFER BETWEEN SPECIES: EFFECTS OF LEGUME PRESENCE AND
SIMULATED GRAZING

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ABSTRACT

Long-term biodiversity field experiments and studies in mesic pastures have revealed positive effects of species richness on productivity and resource use efficiency but detailed information about mechanisms of interaction processes are rare. Thus, we performed a ^{15}N -enriched tracer study (three months in a climate chamber) to investigate N-dynamics between individuals within differently composed communities of mesic grassland species. We investigated how species richness and identity (a grass, forb and legume species) affect N-dynamics and if it is possible to simulate field-effects within a microcosm study. We treated half of the microcosms with “simulated grazing” to investigate how grazing, a common grassland management regime, affects plant-plant interactions.

Higher species richness, but not legume presence, increased short-term N-transfer from ^{15}N -labelled donor to non-labelled receiver individuals. Legume presence increased productivity (NP_{ind}) and nitrogen use efficiency (NUE) of receiver individuals. Results indicate N-facilitation via N-sparing but also the occurrence of short-term bi-directional N-transfer. Species identity had significant effects on the outcome of interactions: the grass profited more from a legume neighbour (higher NP_{ind} , NUE) than the forb, confirming a superior N-acquisition strategy of grasses, which has been found in field experiments. Simulated grazing affected N-transfer differently depending on community composition: N-transfer between individuals increased in monocultures but decreased in mixtures.

In conclusion, we were able to reproduce field-effects in short-term microcosm experiments, which are thus useful to investigate and predict early-successional plant-plant interactions in grassland habitats. Effects of simulated grazing on N-dynamics within the communities were unexpected and are worth further investigations for the emerging field of plant behaviour and kin recognition.

Keywords

Biodiversity, species composition, herbivory, ^{15}N enriched tracer, mesic grassland, *Trifolium pratense*, *Achillea millefolium*, *Phleum pratense*

INTRODUCTION

Diversity of primary producers plays a major role for ecosystem functions like stability, productivity or resource use efficiency (also of higher trophic levels) (Hooper et al. 2005; Balvanera et al. 2006; van Ruijven and Berendse 2009). However, we are still lacking knowledge about the mechanisms how plants interact under different biotic (species richness, composition and assembly) or abiotic (edaphic and climatic conditions, management effects) conditions. Large-scale grassland biodiversity experiments revealed positive relationships between species richness and productivity, resource use efficiency and stability (Tilman et al. 1996; Hector et al. 1999; Roscher et al. 2005; Isbell et al. 2009) although the strength of relationships depends on the abiotic conditions (Grime 1998; Tylianakis et al. 2008; Ma et al. 2010). The insurance hypothesis (McNaughton 1977; Naeem et al. 1994; Yachi and Loreau 1999) summarizes effects of species diversity on stability against disturbances or species invasion and the niche complementarity theory (Berendse 1979; Tilman 1997; Loreau and Hector 2001) summarizes the major explanations for the relationships between biodiversity and ecosystem functioning. Higher spatial and temporal resource complementarity between species for belowground or aboveground resources leads to more effective exploitation and thus higher productivity and stability of more diverse communities. Additionally, facilitation (positive plant-plant interactions) can have strong effects on community performance under resource limited conditions by expanding the realized niche of each species, thus enhancing biodiversity effects even farther (Bruno et al. 2003; Michalet et al. 2006; Brooker et al. 2008). But critical voices also exist, which question positive effects of biodiversity *per se*. They relate the occurrence of such relationships to the inclusion of ecosystem drivers, such as highly productive species, within artificially assembled species pools in experiments; an effect, which is called sampling (or selection) effect (Aarssen 1997; Huston 1997; Leps et al. 2001). Importance of manipulation treatments, including the sampling effect, often increases with decreasing spatial scale (Balvanera et al. 2006). The presence of legume species is often considered as a major component of the sampling effect because legumes can satisfy their own N-demand by biological nitrogen fixation of atmospheric N₂ (BNF) and they often exhibit early, fast and tall growth with high biomass accumulation. On the other hand, legume species can act facilitative on productivity and nitrogen accumulation of neighbouring or subsequent species (N-facilitation) if nitrogen is the main limiting resource. N-facilitation can occur as N-sparing (McNeill and Wood 1990), N-transfer or rhizodeposition of N-rich compounds (Mulder et al. 2002; Spehn et al. 2002; Paynel and Cliquet 2003; Temperton et al. 2007), decomposition of N-rich legume litter (Wang and Bakken 1997; Varvel and Wilhelm

2003) or, as if the legume acts a nurse plant, by providing a better microclimate for saplings of other species (Pugnaire and Luque 2001). But it is important to remark that N-transfer is a bidirectional process which can occur from an N₂-fixing donor to a non-fixing receiver and, to a much lower extent, also in the opposite direction from a non-fixing donor to an N₂-fixing receiver (Hogh-Jensen and Schjoerring 2000; Gylfadottir et al. 2007). Although the transfer from a non-fixing donor might be strongly reduced or is even totally absent under severe N-limitation (Paynel and Cliquet 2003).

Mulder et al. (2002) and Temperton et al. (2007) successfully used the $\delta^{15}\text{N}$ natural abundance method to highlight effects of legume species on neighbouring non-legume species. The $\delta^{15}\text{N}$ natural abundance method uses the ratio of the heavier over the lighter stable isotope of nitrogen ($^{15}\text{N}/^{14}\text{N}$) of a sample and a standard (air) to gain information about the N-source of a plant and the N-dynamics in a system (Shearer and Kohl 1986; Handley and Raven 1992). The $\delta^{15}\text{N}$ natural abundance signal acts as an integrator of the N-dynamics in a system (Robinson 2001) and because of its integrative character, a separation of different nitrogen sources is nearly impossible (except from biological nitrogen fixation of atmospheric N₂). ^{15}N -tracer studies, which use an external, ^{15}N -enriched nitrogen component to follow the path of nitrogen through a system (McNeill et al. 1997; Hertenberger and Waneke 2004), allow assessing different N contributions to the N-status of a plant species. The use of ^{15}N -tracers, applied to the soil, reveal uptake preferences (of N-forms) in different grassland species (Weigelt et al. 2005; Kahmen et al. 2008) or agricultural species (Nasholm et al. 2000). ^{15}N -tracers, applied directly to the plant, provide evidence for N-transfer between species (Hogh-Jensen and Schjoerring 2000; Gylfadottir et al. 2007). However, little information is available about interaction processes, N-transfer and the competitive outcome between legume (N-donor) and different non-legume species (N-receiver) in systems with more than two species and under different management regimes. In respect to the management regime, we know that defoliation, e.g. via grazing, can enhance total biomass production and N-concentration in different grassland species (Sanford et al. 1995; Ayres et al. 2007), change the competitive outcome between species (Barbosa et al. 2009; Rose et al. 2009) and affect community assembly (Olofsson and Shams 2007).

With this study, we aim to resolve effects of community composition (species richness and species identity) on plant-plant interactions, especially on belowground N-transfer between individuals, and how these interactions are affected by a common grassland management (simulated grazing). In addition, we explore the potential to scale up from microcosms to field

experiments. To do this, we conducted a three months microcosm experiment with three different species richness levels, different community compositions and a simulated grazing treatment under controlled environmental conditions in a climate chamber. We used a pulse-chase stable isotope label approach, whereby a ^{15}N -enriched tracer (the pulse) was added to one individual in the system, which was then tracked (chased) in neighbouring individuals. This enabled us to investigate N-transfer from a donor to one or more receiver species, with *either* a legume, a non-legume forb *or* a grass species as donor, and varying receiver species composition. We investigated the effects of the different treatments mainly on individual level to answer the following hypotheses:

- (i) N-transfer will be higher from an N-fixing donor to a non-fixing receiver than between a non-fixing donor-receiver pair.
- (ii) N-transfer will be higher in mixtures than in monocultures because of higher niche complementarity between different species (as observed in the field).
- (iii) Species-specific uptake of transferred N will be modulated by the species composition of the community.
- (iv) N-transfer will increase in response to simulated grazing because of enhanced rhizodeposition following simulated grazing.

MATERIALS AND METHODS

Experimental design

The experiment was conducted from June to August 2008 in a climate chamber under simulated Central European summer conditions and lasted three month (Fig. 1). Light regime was 16/8 hours (light/dark) with twilight-phases of 30 minutes each in the morning and evening. Mean light intensity (measured as photosynthetically active photon flux density, PPFD) above the vegetation canopy was $676 \pm 36 \mu\text{mol}/\text{m}^2/\text{s}$ (± 1 SE); recorded once with a LI-1400 Datalogger and light sensor (LI-COR Bioscience, Lincoln, USA). Temperature regime of 25/15°C (day/night) and air humidity ($\sim 60\%$) were constant during the course of the experiment.

As substrate, we used a mixture of washed sand and agricultural soil from a nearby field (1:1, v/v), sieved with a Retsch sieve (pore size of < 2 mm) to homogenize the substrate and exclude large organic compounds and stones (Retsch GmbH, Haan, Germany). The substrate had pore sizes < 2 mm and total element concentrations of N [%] 0.064 ± 0.002 , C [%] 0.547 ± 0.034 (mean values ± 1 SE) and thus a C:N-ratio of ~ 8.6 . We used 1.5 l square pots for experimental plant communities (= microcosms). Microcosms were placed in a random distribution on six movable tables – microcosms (on tables) and tables (in the chamber) were rotated weekly to prevent confounding block/chamber or edge effects. Plants were watered manually with a mixture of rain and tap water every second day.

Main focus of the experiment was to follow the path of nitrogen from donor to receiver individuals and how species composition, species identity and an applied treatment (simulated grazing) affect N-transfer from donor to receiver individuals. For the experimental plant communities, we used three species from three functional groups and grew them in three species richness level (Fig. 1). Species used were: *Trifolium pratense* L. (tri; N₂-fixing forb, hereafter: legume), *Achillea millefolium* L. (ach; non-fixing forb, hereafter: forb) and *Phleum pratense* L. (phl; grass) (Oberdorfer 2001); to avoid confusion between the legume and the grass species, we will refer to the genus name in the following. Species richness levels were: monocultures (mono), 2-species-mixtures (2-mix) and 3-species-mixtures (3-mix); Figure 1 gives some examples of community compositions. 2-species-mixtures were available with and without the legume species as donor; whereas 3-species-mixtures always contained the legume species as donor. For each community we transplanted 5-6 seedlings of approximately 2-4 cm height at the beginning of the experiment in a star-like fashion: 1 individual in the

centre of the microcosm (^{15}N labelled donor) and 4-5 individuals (unlabelled neighbouring receivers) at the same distance to the central individual and to every neighbour around the donor individual (Fig. 1).

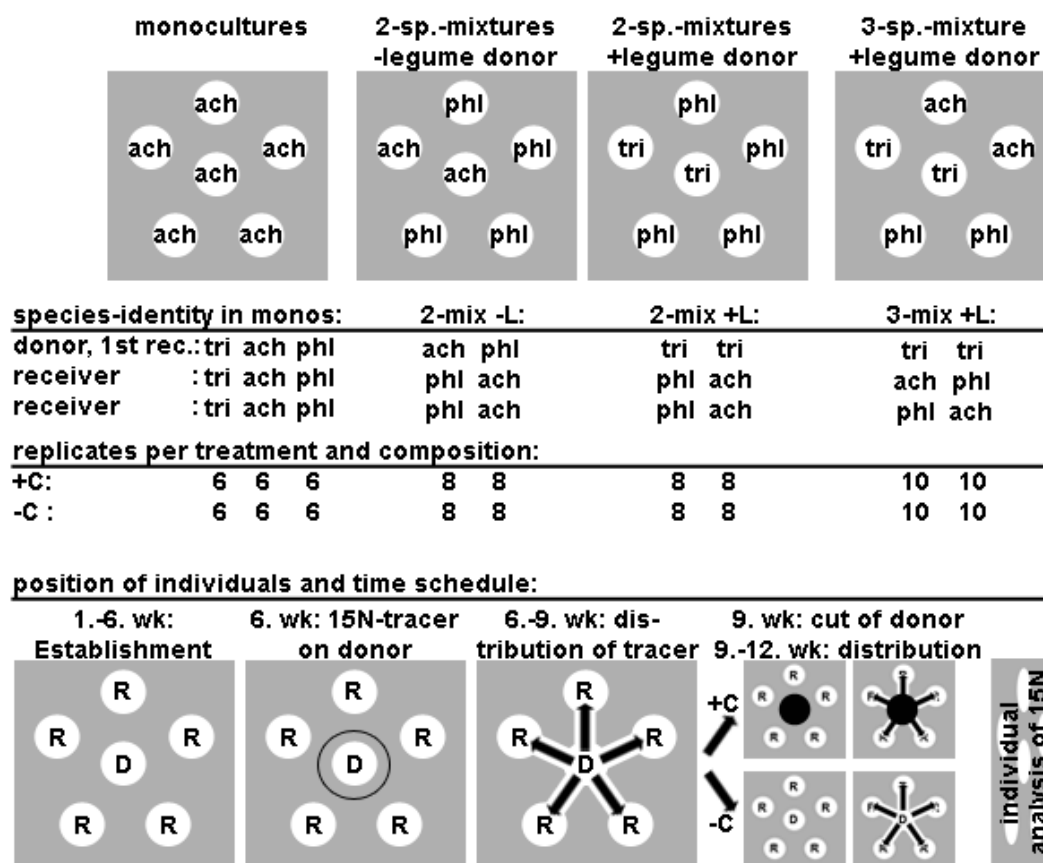


Figure 1 Experimental design of the microcosm study; indicated are examples for community compositions in three species richness levels (monoculture, 2-species-mixture without and with a legume donor and 3-species-mixture with a legume donor), species identity of donor and receiver individuals in the communities (*Trifolium pratense* L. (tri; legume), *Achillea millefolium* L. (ach; forb) and *Phleum pratense* L. (phl; grass)) with the number of replications and the time schedule for the experiment. It started in early June (03.06.2008) and ended with the harvest in the end of August (26.08.2008); ^{15}N -labelling procedure on donor individuals (D) took place during the 6th weeks (09.-16.07.2008), followed by 3 weeks of tracer distribution from donor to receiver individuals (R) until application of simulated grazing of donor individuals in the 9th week (01.08.2008; with simulated grazing/cutting = +C, without = -C) and again a three-weeks time span to allow for an impact of simulated grazing on ^{15}N -transfer.

To identify N-transfer from donor to receiver individuals, we applied 1 ml of a ^{15}N enriched label-solution (0.5 % (v/v) of 99 atom% ^{15}N -enriched urea (Campro Scientific GmbH, Berlin, Germany) diluted in 2 ml Eppendorf vials[®] in deionised water) via the leaf to the donor individual based on the method described by McNeill et al. (1998). Leaf labelling was done during the 6th week of the experiment (Fig. 1). To facilitate uptake of the label-solution into

leaves of the donor, leaf tips (of one leaf for *Trifolium* and *Achillea* and of two leaves for *Phleum*) were cut and the upper 2-3 cm of the leaf/leaves were submerged for one week in the solution. Loss of the enriched solution by evaporation or transfer to non-target compartments (receivers, substrate) as well as dilution by irrigation water was prevented by carefully sealing the opening with the leaf/leaves in with a putty-like pressure-sensitive adhesive substance (Blue tack[®]). Receiver individuals were not labelled. Remains of the label-solution together with the labelled leaves were removed after one week.

We applied the simulated grazing treatment in the 9th week (Fig. 1) by cutting the whole shoot biomass of the donor individual 0.5-1 cm above the substrate. Simulated grazing was applied to half of the microcosms; this treatment will be denoted '+C' in the following. The remaining pots were not cut (control) and will be denoted '-C'.

Response parameters

To gain information on individual productivity, all harvesting activities were done separately for every individual per species per pot. Donor individuals from the simulated grazing treatment (+C) were cut and then oven-dried (at 60°C for 60 h) during the 9th week. Regrowth of the cut donor individuals was followed by measuring length and ground cover of regrown parts weekly, which were then removed and oven-dried. All parts of donor individuals were stored until final harvest, which took place at the end of August 2008 (Fig. 1). During the final harvest, remaining donor individuals from control communities (-C) and all receiver individuals were cut above the substrate and oven-dried. At the final harvest, we took an unspecific root sample (and oven-dried it) from every microcosm, because separation of roots on species level was not possible. Dry weight per individual was determined to gain aboveground net production of individuals (NP_{ind} [g]). NP_{ind} of the donor (including regrown parts for +C communities) and normally harvested receiver individuals was summed up for total community biomass per microcosm.

For determination of N-dynamics between donors and receivers, the measurement of N-parameters in samples was done separately for the unspecific root sample, the donor species and for each *receiver species* per microcosm. Receiver individuals were pooled per species per microcosm to obtain one samples per receiver species; we validated this way to conduct analyses of N-parameters by measuring individuals from a subset of microcosms separately (Table 1).

Table 1 Within species variation of separately measured receiver individuals per microcosm in a subset of communities; we measured these unpooled individuals to validate the use of pooled receiver individuals to gain information about N-dynamics in populations. Indicated are N-concentration [%], enrichment of ^{15}N [atom%excess] and transfer of ^{15}N from donor to receivers [%] (^{15}N -transfer) for species richness levels monoculture, 2-species-mixture and 3-species-mixture of the species *Trifolium pratense*, *Achillea millefolium* and *Phleum pratense*. Values are means (from n individuals per microcosm per mixture) \pm 1 standard deviation of the mean (SD).

	receiver species	n	N [%]	SD	^{15}N [atom% excess]	SD	^{15}N -transfer [%]	SD
monoculture								
	<i>Trifolium</i>	5	2.83	0.35	0.0025	0.0006	3.08	0.76
	<i>Achillea</i>	5	1.04	0.15	0.1010	0.0034	3.03	1.01
	<i>Phleum</i>	5	0.57	0.06	0.0181	0.0014	0.57	0.04
2-species-mixture								
	<i>Trifolium</i>	1	2.74		0.0021		0.50	
	<i>Achillea</i>	4	0.90	0.09	0.0110	0.0012	2.66	0.29
	<i>Trifolium</i>	1	2.81		0.0022		0.55	
	<i>Phleum</i>	4	0.93	0.10	0.0256	0.0146	6.42	3.67
	<i>Achillea</i>	1	0.95		0.1451		0.47	
	<i>Phleum</i>	4	0.77	0.04	0.0733	0.0267	0.24	0.09
	<i>Phleum</i>	1	1.07		0.0799		2.52	
	<i>Achillea</i>	□	0.92	0.02	0.0354	0.0153	1.12	0.48
3-species-mixture								
	<i>Trifolium</i>	1	2.80		0.0029		1.25	
	<i>Achillea</i>	2	1.03	0.27	0.0144	0.0005	6.27	0.21
	<i>Phleum</i>	2	1.31	0.07	0.0266	0.0042	11.63	1.85
	<i>Trifolium</i>	1	2.99		0.0173		0.97	
	<i>Phleum</i>	2	1.21	0.08	0.0267	0.0071	1.50	0.40
	<i>Achillea</i>	2	1.06	0.02	0.0130	0.0053	0.73	0.30

For measurement of N-parameters, shoot and root samples were ground to fine powder using a Retsch ball mill MM 301 (Retsch GmbH, Haan, Germany) with stainless steel devices. Analyses of N-concentration [%] and of ^{15}N -enrichment [atom % excess] was done with an ANCA-SL 2020 EA-IRMS (element analyser – isotope ratio mass spectrometer; SerCon Ltd. (formerly Europa & PDZ Ltd.), Crewe, UK). ^{15}N -enrichment [atom % excess] was calculated from the isotopic composition (^{15}N and ^{14}N) in a sample: the occurrence of ^{15}N -isotopes [atom %] in a sample minus the natural occurrence of ^{15}N -isotopes [atom %] in the atmosphere, which is 0.3663 %, resulted in the value for ^{15}N -enrichment [atom % excess]. For readability of results, we will use the shortened [at%ex] as unit for ^{15}N -enrichment. We calculated the amount of ^{15}N -transfer [%] from donors to receivers using the total ^{15}N -enrichment [at%ex] of the donor (as 100 %) and the ^{15}N -enrichment [at%ex] of receivers (as x % of enrichment of the donor) at the time of harvest. The ^{15}N transfer [%] value was used to determine how much of ^{15}N from label was transferred to receivers during the course of the experiment.

We inserted a decomposition standard near the edge of every pot to test if community composition or presence of a certain functional group had an effect on belowground decomposition. The standard consisted of a strip (1 x 5 cm, made from normal laboratory cellulose filter paper) which was fixed in a stainless steel grid to facilitate recovery at the end of the experiment. We estimated degradation of the decomposition standard [%] as a rough estimate for belowground turnover.

We stained a subsample of randomly chosen fresh roots from 18 microcosms (6 from mono, 8 from 2-mix and 4 from 3-mix) with a trypan blue root staining (Phillips and Hayman 1970) to assess the infection with mycorrhizal fungi (MF). We checked for MF hyphae in- and outside the root cortex and for the formation of vesicles and arbuscules inside the tissue by microscopic observation but did not determine percentage of infected root length, as root subsamples could not be ascribed to species.

Statistics

We analysed the data on community level and on species, separated in donors and receivers, to gain information about the overall functioning of communities as well as about the interactions between different individuals within a community. Prior to statistical analyses, all data and data subsets were tested for homogeneity of variance (Levene's test) and normality (Kolmogorov-Smirnov test, Q-Q-Plots) and transformed, if assumptions were not met. Then, data were analysed by the use of general linear models. Generally, we used ANCOVA (type I sum of squares); only for the analysis of total community biomass, we used an ANOVA without a covariable. ANCOVA (type I sum of squares) was conducted with biomass of donor individual (NP_{donor}) as covariable, different response parameters (N-concentration, N-content, ^{15}N -transfer) as dependent variable and experimental treatments as fixed factors (Table 2). The fitting order of factors was changed to identify which factor had most impact on response parameters. We conducted ANCOVA for all data together (over all species richness levels) and for every species richness level separately. We used least-significant-difference test (LSD) as a post-hoc test when significant differences were found between treatments. All statistical analyses were conducted in SPSS 11 by SPSS Inc., USA.

We tested for effects of the experimental treatments (factors) species richness level (SR: 3 levels; mono, 2-mix, 3-mix), legume presence (L: 2 levels; without and with *Trifolium*) and simulated grazing (C: 2 levels; without and with cutting of donor individual) and additionally

species identity of donor and receiver individuals (SI: 3 levels, one for each species). Individual response parameters (dependent variables) were: dry weight for net biomass production of individuals (NP_{ind} [g], square root transformation), N-concentration [%] (data+1, logarithmic transformation, lg_{10}), N-content [mg] ($NP_{ind} \times N$ [%], data+1, logarithmic transformation, lg_{10}) and ^{15}N -transfer from donor to receiver individuals [%] (data+5, inverse transformation). Community response parameters (dependent variables) were: degradation of decomposition standards [%] (arcsine transformation), dry weight for net biomass production of communities [g] (no transformation) and total N-content [mg] (square root transformation). Data of mycorrhizal fungi infection of communities were not analyzed statistically because it was a pure descriptive parameter.

RESULTS

Individual and population response

Donors

Biomass production (NP_{donor}) and N-concentration of donor individuals were mostly not significantly different from receiver individuals although donors were generally slightly smaller and had a higher N-concentration in their tissue than receivers (data not shown). Total ^{15}N -enrichment of donor individuals varied strongly from 0.0089 to 36.6155 [at%ex]. Enrichment was 16.81 ± 2.02 ^{15}N at%ex for *Achillea* (0.2306 to 36.6155 at%ex, $n = 28$) and 4.75 ± 0.78 ^{15}N at%ex for *Phleum* (0.7697 to 20.7490 at%ex, $n = 28$). Enrichment of *Trifolium* donor individuals was 0.75 ± 0.11 ^{15}N at%ex (0.0089 to 4.7726 at%ex, $n = 84$). Species-specific values for enrichment were mean values ± 1 standard error of the mean and, in brackets, the minima and maxima per species with indication of the number of donors over all species richness levels. A kind of dilution effect occurred and lower ^{15}N -enrichment [at%ex] in donor individuals correlated with higher individual biomass (NP_{donor}) (Pearson correlation coefficient $\rho = 0.612$, $p < 0.001$), which could be described with an inverse (exponential decay) regression ($r^2 = 0.705$, $p < 0.001$; variables were square root transformed for both analyses).

Receivers

Effects of legume presence and species composition

H1: N-transfer will be higher from an N-fixing donor to a non-fixing receiver than between a non-fixing donor-receiver pair.

Direct comparison between ^{15}N -transfer from a legume vs. a non-legume donor individual was possible for different species compositions in 2-species-mixtures. We used ANCOVA models (type I sum of squares) to investigate the effect of species identity (forb, grass or legume species) of the donor individual (SI_{donor}) and of receiver individuals (SI_{receiver}), fitted with the effect of donor individual's biomass (NP_{donor}) as covariable (Table 2a). We found that effects of receiver species identity were as strong as effects of NP_{donor} for ^{15}N -transfer and for the other response parameters whereas effects of species identity of donor individuals were less strong (lower F-values). Species identity of donor individuals affected biomass production of receivers (NP_{ind}), N [%] and N-content significantly but not ^{15}N -transfer. In

general, ^{15}N -transfer was highest from *Trifolium* as a donor and lowest from *Achillea* as a donor but this trend was not statistically significant (LSD *Trifolium* > *Phleum* $p = 0.843$ and *Phleum* > *Achillea* $p = 0.495$).

H2: N-transfer will be higher in mixtures than in monocultures because of higher niche complementarity between different species (as observed in the field).

Transfer of ^{15}N from donors to receivers increased from monocultures to 3-species-mixtures (Fig. 2) whereas net biomass production of receiver individuals (NP_{ind}), N [%] and N-content [mg] were higher in monocultures and 3-species-mixtures than in 2-species mixtures (data not shown).

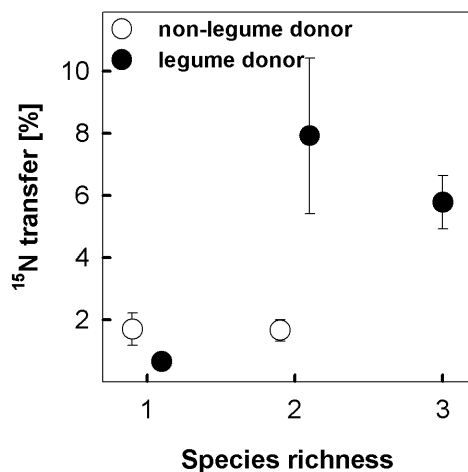


Figure 2 Effect of species richness level and legume presence (N_2 -fixing donor species) on ^{15}N -transfer* to receivers, averaged over all species in the species richness levels monocultures (1), 2-species-mixtures (2) and 3-species-mixtures (3) without (\circ /open circles) or with (\bullet /closed circles) presence of *Trifolium pratense* as donor species. Due to the experimental design, 3-species-mixtures always contained *Trifolium* as donor species.

* ^{15}N -transfer [%] was measured as ^{15}N -enrichment [atom%excess] in non-labelled neighbouring receiver individuals in relation to ^{15}N -enrichment [atom%excess] in ^{15}N -labelled donor individual after the harvest.

Analyses over all species richness levels together showed strong effects of net biomass production of donor individuals (NP_{donor}) on all four individual response parameters (Table 2b). Most variation in ^{15}N -transfer was explained by NP_{donor} and the species richness level (Table 2b, Fig. 2) whereas most variation in NP_{ind} , N [%] and N-content was explained by NP_{donor} and legume presence (higher F-values for NP_{donor} and L than for SR, Table 2b). Across all species richness levels, legume presence increased NP_{ind} , N [%] and N-content [mg] in receivers (data not shown) but not ^{15}N -transfer from donors to receivers. Significant interactions between species richness level and legume presence showed that the effect of *Trifolium* increased with increasing diversity. Within species richness levels (Fig. 3), strong effects of donor individual biomass (NP_{donor}) on all four response parameters of receivers

remained but effects of receiver's species identity were often even stronger (Table 2c). Especially ^{15}N -transfer in monocultures and 2-species-mixtures depended more on the identity of the receiver than on NP_{donor} , whereas in the 3-species-mixtures no effect of NP_{donor} occurred because *Trifolium* was always the donor, which produced comparable high NP_{donor} in all microcosms. Although figure 3 suggested a pronounced positive legume effect, especially on ^{15}N -transfer from donor to receiver individuals, this legume effect was possibly cancelled out by the strong impact of NP_{donor} .

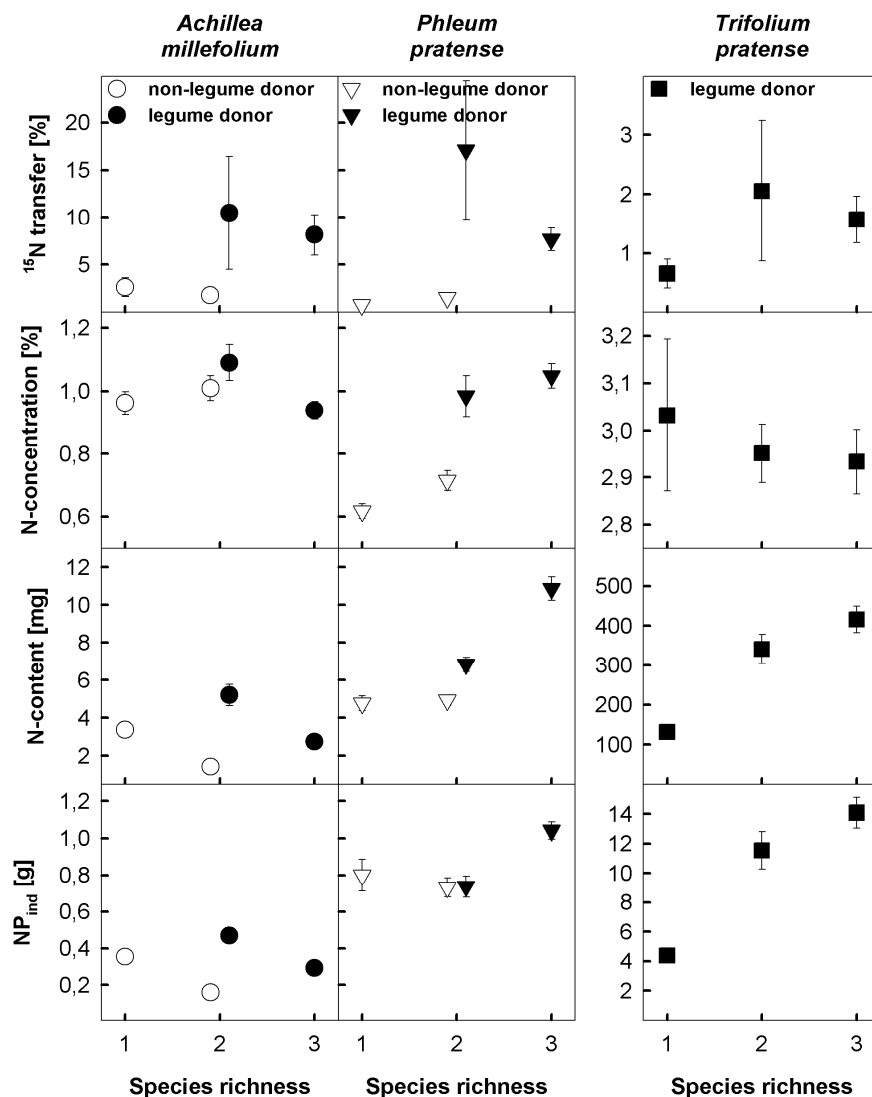


Figure 3 Effect of species richness level and **legume presence** (N_2 -fixing donor species) on species-specific response parameters (^{15}N -transfer, N [%], N [mg] and NP_{ind}) in receiver individuals in the species richness levels monocultures (1), 2-species-mixtures (2) and 3-species-mixtures (3) without (○/open circles) or with (●/closed circles) presence of *Trifolium pratense* as donor. Response parameters were ^{15}N -transfer [%] from ^{15}N -labelled donor to non-labelled receiver individuals*, N-concentration [%], N-content [mg] and net biomass production of receiver individuals (NP_{ind}) [g], dry weight. *details see Fig. 2

H3: Species-specific uptake of transferred N will be modulated by the species composition of the community.

Species identity of receiver individuals had significant effects on response parameters in all species richness levels (Table 2c). *Trifolium* always received the least ^{15}N , irrespectively of the species richness level (Fig. 3). In monocultures, most ^{15}N was transferred between donor and receiver individuals of *Achillea*. In 2-species-mixtures, *Phleum* received significantly more ^{15}N from donor individuals (no separation in legume and non-legume donors) than *Achillea* (LSD $p = 0.038$). In direct competition between forb and grass individuals in 3-species-mixtures, *Phleum* received non-significantly more ^{15}N from donor individuals than *Achillea* (LSD $p = 0.449$) but used this N more effectively for higher NP_{ind} , N [%] and N-content (LSD $p \leq 0.038$) (Fig. 3).

Effect of simulated grazing

H4: N-transfer will increase in response to simulated grazing because of enhanced rhizodeposition following simulated grazing.

Simulated grazing affected ^{15}N -transfer from donor to receiver individuals within and across species richness levels. Analyses of regrown donor parts showed, that most ^{15}N was used for internal N-remobilization to sustain regrowth of the cut donor individual, ca. 56 % of donor individual ^{15}N at%ex was recycled internally (Fig. 4).

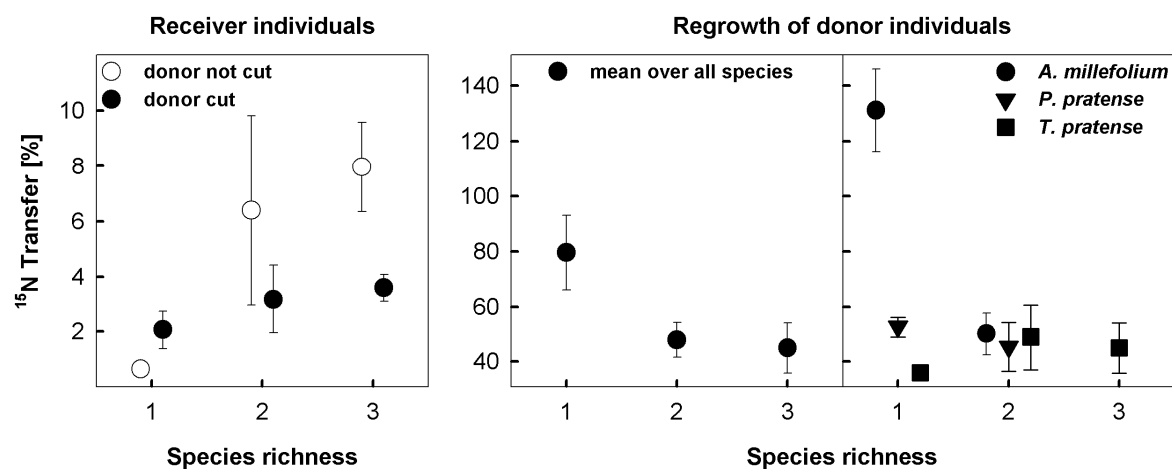


Figure 4 Effect of species richness level and **simulated grazing** (cutting of donor) on overall ^{15}N -transfer [%] from donor to receiver individuals (mean values over all species per diversity level) and internal remobilization of ^{15}N to regrown parts of the cut donor individual in the species richness levels monocultures (1), 2-species-mixtures (2) and 3-species-mixtures (3) without (○/open circles) or with (●/closed circles) cutting of donors.

Simulated grazing stimulated ^{15}N -transfer in monocultures but decreased the transfer in mixtures; an effect that was detected across all individuals (Fig. 4, LSD –C vs. +C: $p = 0.038$, 0.662 and 0.035 in mono, 2-mix and 3-mix, respectively) and separately for the three species (Fig. 5). Across all species richness levels, the effect of simulated grazing on ^{15}N -transfer was only minor (Table 2d) compared to effects of net biomass production of donor individuals (NP_{donor}), species identity of receivers ($\text{SI}_{\text{receiver}}$) and species richness levels *per se* (Table 2a-c), as F-values of factors in relation to the fitting order of the factors in ANCOVA models (type I sum of squares) showed. This is reflected in the finding, that significant differences in ^{15}N -transfer occurred only for *Achillea* (LSD $p < 0.05$ in mono and 3-mix) but not for *Phleum* or *Trifolium* (Fig. 5). Generally, simulated grazing had no effect on NP_{ind} , N-concentration

and N-content, except for a significantly decreased NP_{ind} of *Phleum pratense* receivers after cutting of donor individuals in 3-species-mixtures.

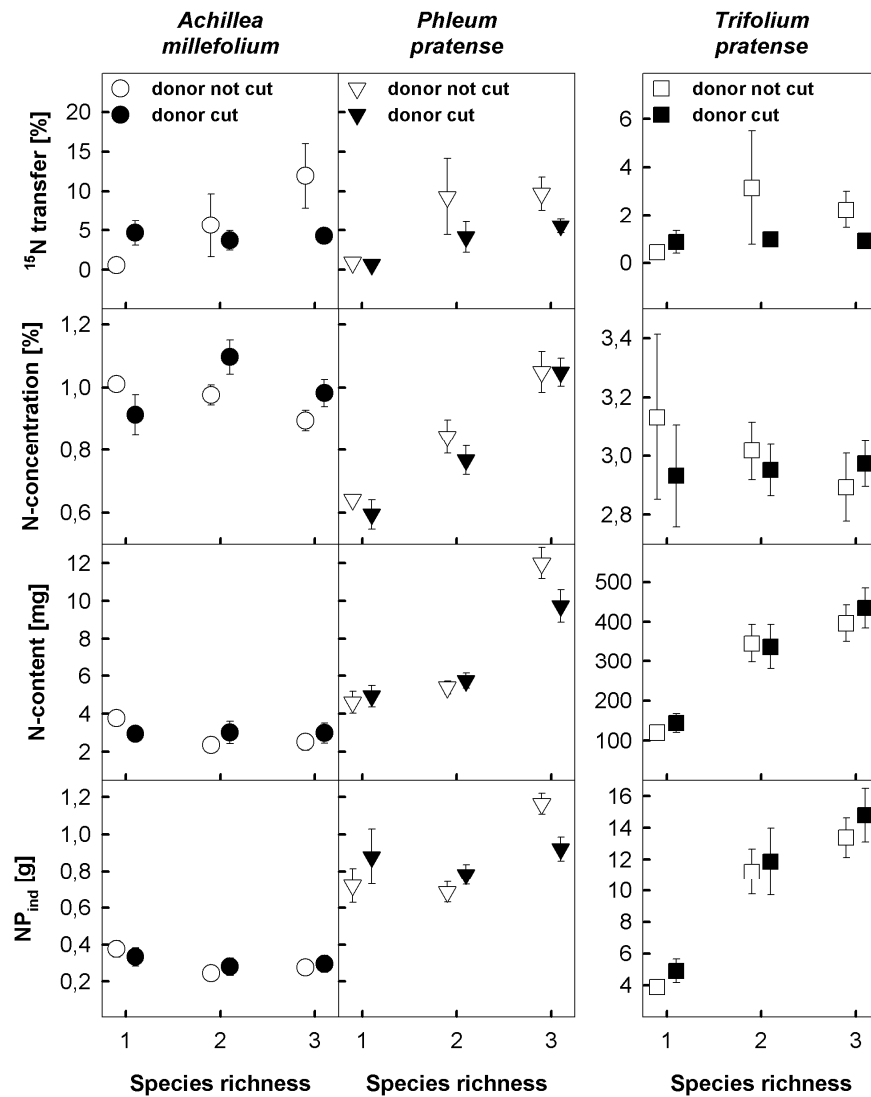


Figure 5 Effect of species richness level and **simulated grazing** (cutting of donor) on species-specific response parameters (^{15}N -transfer, N [%], N [mg] and NP_{ind}) in receiver individuals in the species richness levels monocultures (1), 2-species-mixtures (2) and 3-species-mixtures (3) without (\circ /open circles) or with (\bullet /closed circles) cutting of donors. Response parameters were ^{15}N -transfer [%] from ^{15}N -labelled donor to non-labelled receiver individuals*, N-concentration [%], N-content [mg] and net biomass production of receiver individuals (NP_{ind}) [g], dry weight. *details see Fig. 2

hypothesis: (i) N-transfer will be higher from legume than from non-legume donors
(iii) species-specific uptake will vary with community composition

hypothesis: (i) N-transfer will be higher from legume than from non-legume donors
(ii) N-transfer will be higher in mixtures than in monocultures

hypothesis: (ii) N-transfer will be higher in mixtures than in monocultures
(iii) species-specific uptake will vary with community composition

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L x SI_{receiver}	4	84.30	< 0.001	210.18	< 0.001	143.00	< 0.001	15.83	< 0.001
L and SI_{receiver}	0	n.a.							
3-species-mixtures:									
NP_{donor}	1	2.51	0.116	< 0.01	0.957	0.283	0.596	0.154	0.696
SI_{receiver}	2	387.09	< 0.001	511.86	< 0.001	968.88	< 0.001	28.08	< 0.001
L and SI_{receiver} x L	0	n.a.							

hypothesis: (iv) Simulated grazing will enhance N-transfer

d) test group: all species richness levels together, all species together

(covariable: fitted first; interactions: fitted in the same order at the end of each model – both were only given once because of reasons of readability)

factor	d.f.	NP _{ind}		N [%]		N [mg]		¹⁵ N-transfer	
		F	p	F	p	F	p	F	p
NP_{donor}	1	156.50	< 0.001	433.59	< 0.001	455.52	< 0.001	43.69	< 0.001
SR	2	1.95	0.144	12.94	< 0.001	12.22	< 0.001	8.93	< 0.001
L	1	150.77	< 0.001	509.88	< 0.001	417.97	< 0.001	0.13	0.723
SI_{receiver}	2	525.08	< 0.001	934.20	< 0.001	1015.13	< 0.001	62.82	< 0.001
C	1	0.01	0.912	< 0.01	0.971	0.06	0.809	0.64	0.426
C	1	8.39	0.004	20.38	< 0.001	17.13	< 0.001	0.08	0.784
SI_{receiver}	2	581.78	< 0.001	1182.22	< 0.001	1216.57	< 0.001	59.48	< 0.001
L	1	2.65	0.105	17.06	< 0.001	15.82	< 0.001	24.66	< 0.001
SR	2	15.12	< 0.001	1.13	0.324	3.33	0.037	0.28	0.752
L	1	140.86	< 0.001	366.03	< 0.001	339.29	< 0.001	1.75	0.187
C	1	0.05	0.830	0.26	0.613	0.49	0.483	0.41	0.521
SR	2	6.92	0.001	84.91	< 0.001	51.56	< 0.001	8.11	< 0.001
SI_{receiver}	2	525.06	< 0.001	934.02	< 0.001	1014.91	< 0.001	62.94	< 0.001
SI_{receiver}	2	585.83	< 0.001	1191.72	< 0.001	1224.85	< 0.001	59.41	< 0.001
C	1	0.29	0.593	1.38	0.241	0.56	0.455	0.26	0.636
SR	2	16.11	< 0.001	1.39	0.250	8.61	< 0.001	4.34	0.014
L	1	0.67	0.412	16.54	< 0.001	5.25	0.023	16.53	< 0.001
SR x L	1	45.64	< 0.001	1.03	0.311	25.58	< 0.001	0.07	0.790
SR x SI_{receiver}	3	2.47	0.063	13.70	< 0.001	7.25	< 0.001	1.88	0.133
SR x C	2	0.18	0.837	1.48	0.229	0.02	0.985	3.87	0.022
L x SI_{receiver}	1	1.91	0.168	5.16	0.024	7.97	0.005	1.99	0.160
L x C	1	< 0.01	0.964	1.92	0.167	0.11	0.737	0.14	0.710
SI_{receiver} x C	2	0.12	0.887	1.97	0.141	0.33	0.723	0.91	0.402

Microcosms and communities

Total biomass [g] and nitrogen content [mg] per pot increased with increasing species richness, which is mainly due to presence of *Trifolium* (Fig. 6). Individuals of *Trifolium* produced always most biomass and had highest N-concentrations (Fig. 3), thus, their relative contribution to community biomass and N-content was high. We analysed data with ANOVA models (type I sum of squares) and found that both species richness levels (SR) and legume presence (L) had highly significant effects ($F_{1-2,134} > 100$, $p < 0.001$) on community biomass and N-content, if fitted first in the model. But if legume presence (L: $F_{1,134} = 1230.376$, $p < 0.001$) was fitted before SR, the effect of SR was not longer significant ($F_{2,134} < 0.600$, $p > 0.500$). Simulated grazing (fitted in the model first or third) reduced total biomass of communities (C: $F_{1,134} > 6$, $p < 0.02$, data not shown).

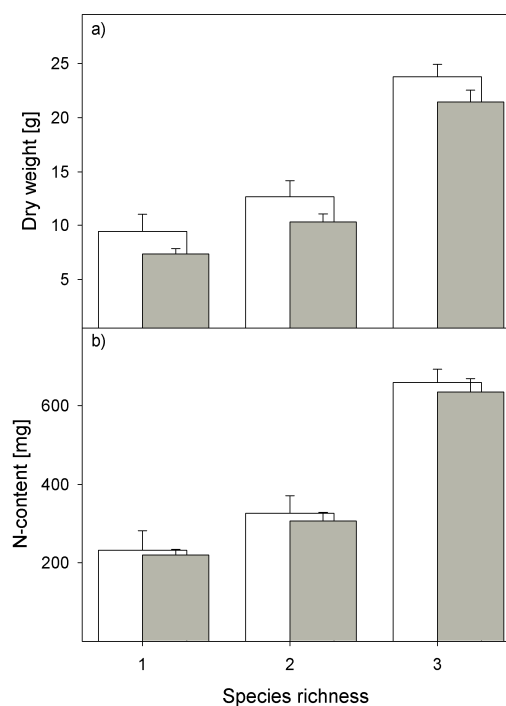


Figure 6 Effect of species richness level and legume presence in communities on (a) total biomass, dry weight [g] and (b) total N-content [mg] of communities per microcosm in different species richness levels (monocultures, 2-species- and 3-species-mixtures). White bars indicate values for whole communities (sum of all individuals, no separation in donor and receiver), grey bars indicate the relative contribution of highly productive *Trifolium pratense* individuals (donor and receiver individuals) within these communities for community responses. Values are means \pm 1 standard error of the mean.

We found vesicles, arbuscules, internal and external hyphae of mycorrhizal fungi (MF), which are typical structures denoting vesicular-arbuscular mycorrhiza (VAM), in 17 out of 18 root samples. We found infection with MF in $40.8\% \pm 26.5$ (SD) of stained roots per sample.

Species richness level, legume presence and simulated grazing had no effect on the degradation of decomposition standards across all species richness levels. Within species richness level, analyses showed a significantly higher degradation in monocultures of *Trifolium pratense* than in monocultures of the other two species ($F_{1,36} = 4.9$, $p = 0.034$) (Fig. 7). No statistically significant interaction between legume presence and simulated grazing occurred. We thus assumed that below ground turnover rates were not affected by species richness levels and no correction for different turnover rates had to be applied.

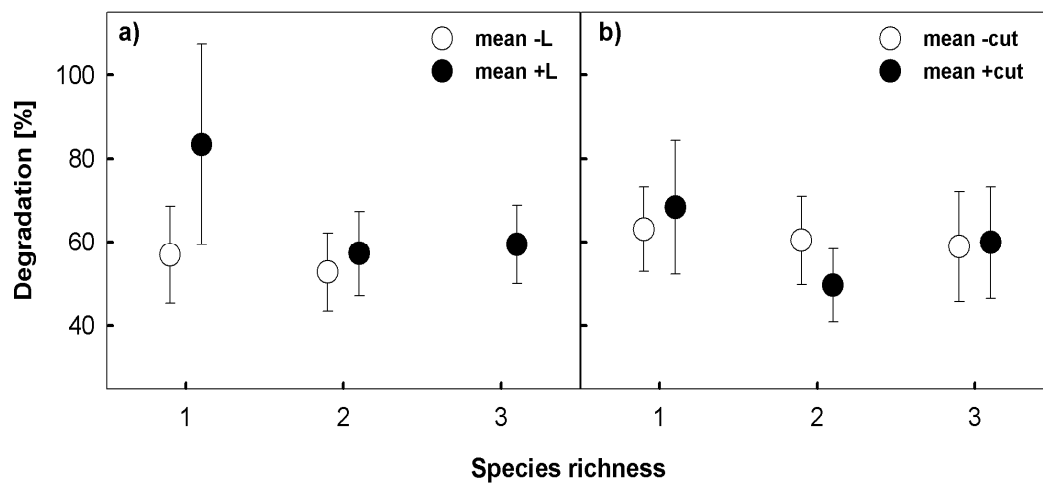


Figure 7 Effect of legume presence and simulated grazing on degradation of decomposition standard in communities along the species richness levels (monocultures, 2-species- and 3-species-mixtures), given are mean values (± 1 standard error of the mean, replicates are given in Fig. 1) for (a) degradation [%] in communities without (\circ /open circles) or with (\bullet /closed circles) *Trifolium pratense* and (b) degradation [%] in untreated communities (\circ , open circles) and in communities treated with simulated grazing (\bullet , closed circles) of donor individuals.

DISCUSSION

Individual level

Effects of legume presence and species composition

H1: N-transfer will be higher from an N-fixing donor to a non-fixing receiver than between a non-fixing donor-receiver pair.

The first hypothesis, that ^{15}N -transfer will be higher from a legume than from a non-legume donor individual, was not found to be true because (apart from a pronounced positive legume effect in Figs 2, 3). Statistical analyses revealed only minor effects of donor individual's species identity for ^{15}N -transfer, because effects were overruled by effects of the biomass of donor individuals (effects of NP_{donor} and $\text{SI}_{\text{receiver}}$, see Table 2a). Although significant interactions, if fitted first in the type I sum of squares ANCOVA models, between species richness level and legume presence ($\text{SR} \times \text{L}$; Table 2b), between receiver's species identity and legume presence ($\text{L} \times \text{SI}_{\text{receiver}}$; Table 2c) and between receiver's and donor's species identity ($\text{SI}_{\text{receiver}} \times \text{SI}_{\text{donor}}$; Table 2a) suggested that the presence of the key species *Trifolium pratense* across and within the species richness levels altered N-transfer between species. Legume presence affected individual net biomass production (NP_{ind}), N-concentration and N-content in receiver individuals positively; this showed an apparent short-term facilitative effect of legume presence. Positive legume effects on biomass and N-accumulation have been observed frequently in experimental grassland communities in the field (Lee et al. 2003; Temperton et al. 2007). Facilitative legume effects are related to two processes: (i) N-transfer from legume to neighbour and (ii) N-sparing (increased amount of soil-N for non-legumes if a resident legume species relies more on atmospheric- N_2 than on soil N-resources). Legume effects often increase with time because of an accumulation of N-rich legume litter (Hogh-Jensen and Schjoerring 1997; Mulder et al. 2002) or other factors, which increase complementarity effects (Marquard et al. 2009). Here, the facilitative legume effect is most likely related to short-term N-sparing and not driven by increased N-transfer as Temperton et al. (2007) also report from a study within a semi-natural grassland field experiment. We were able to show that N-transfer, from legume and non-legume donors, occurred during a 12 week pot experiment and that is not *per se* a slow process as stated by Ledgard & Steele (1992), but that N can be transferred between species of different functional identities within time periods of 20-30 days in accordance to the field-study from Gylfadottir et al. (2007). Short-term positive legume effects were not only related to N-sparing; we found a trend for higher ^{15}N -

transfer from legume donor individuals; but the difference in size of non-legume vs. legume donors did not allow for a comparison of differences in N-transfer between legume and non-legume donors of approximately the same biomass within this study.

H2: N-transfer will be higher in mixtures than in monocultures because of higher niche complementarity between different species (as observed in the field).

We found higher ^{15}N -transfer from donor to receiver individuals in more diverse communities (Table 2b) confirming the second hypothesis. This finding is in accordance with the hypothesis that an early species saturation (3-9 species) occurs, if only one function (here, the response parameter ^{15}N -transfer) is investigated (Schwartz et al. 2000; De Boeck et al. 2007); although recent studies emphasise the importance of higher species richness for the maintenance of multifunctionality of communities and ecosystems (Hector and Bagchi 2007; Marquard et al. 2009; Zavaleta et al. 2010). Higher species richness, although here in a very small range, probably led *per se* to higher niche complementarity as it has been found in large-scale, long-term field experiments (e.g. Tilman et al. 2001; Roscher et al. 2005; Hector et al. 2007; Marquard et al. 2009; van Ruijven and Berendse 2009). The beneficial effect of higher species richness was not overruled by the dilution effect of higher net biomass production of donor individuals (NP_{donor}) on ^{15}N -enrichment [at%ex] in donors, which had a feedback on ^{15}N -transfer [%] to receivers. Whereas effects of NP_{donor} overruled positive effect of legume donors, which only *looked* strong in graphical data presentation (Figs 2, 3) but were not statistically significant (Table 2b, c).

H3: Species-specific uptake of transferred N will be modulated by the species composition of the community.

Species composition had strong effects on interactions between different species (Fig. 3, Table 2a, c) as predicted by the third hypothesis. We were able to show, that the grass species *Phleum* competed more effectively for soil N-resources and for ^{15}N released from donor individuals than *Achillea*. The effect was consistent in 2- and 3-species-mixtures. Grass species are more effective in capturing extra N compared to forb species as found in long-term grassland field experiments (Oelmann et al. 2007; Temperton et al. 2007). This is in accordance with the finding that grasses have a finer, more dense root systems (Craine et al.

2002) and exploit resources faster and more effectively (Šmilauerová and Šmilauer 2010) than forb species. We showed that also in the short-term the grass species benefited more from *Trifolium* than the forb, if all three species grew together, which indicate a better nitrogen use efficiency (NUE; because with nearly the same amount of ^{15}N [%] transferred, a stronger positive biomass and N accumulation [mg] effect was achieved).

Although *Achillea* receivers competed successfully for released ^{15}N with the grass species, they could not implement this surplus N into increased growth during the time span of this experiment. It should be noted that the overall growth of *Achillea* and *Phleum* was lower than that of *Trifolium* (Fig. 3). Nevertheless we found positive legume effects. Especially the forb produced very little biomass per individual compared to other studies (Kowal and Pic 1979; Johnston and Pickering 2007) and its normal productivity in nature where it grows up to 100 cm (Oberdorfer 2001).

Effect of simulated grazing

H4: N-transfer will increase in response to simulated grazing because of enhanced rhizodeposition following simulated grazing.

We could neither clearly confirm nor reject the fourth hypothesis which predicted higher N-transfer after simulated grazing due to higher rhizodeposition from belowground parts of cut donor individuals. Simulated grazing resulted in higher ^{15}N -transfer [%] from cut donors to neighbouring receivers in monocultures but to a decrease of transfer in mixtures compared to control communities (donors not cut; Figs 4, 5). Ayres et al. (2007) found a pronounced increase in ^{15}N -transfer from clover to roots of ryegrass after defoliation (but no significant effect in shoots of the grass species) whereas Paterson et al. (2005) found decreased exudation of isotopic C-tracers from *Festuca rubra* roots 2-4 days after defoliation. Ayres et al. (2007) relate the immediate strong increase in N-transfer to higher N-releases from the defoliated clover plant via direct pathways (exudation, mycorrhiza) although they do not exclude higher indirect effects (decomposition) because they observed higher microbial biomass after defoliation. Although we did not investigate species-specific root samples or microbial biomass in this study, we found a contrary pattern of decreased inter-specific ^{15}N -transfer after simulated grazing. Evidence for higher rhizodeposition with subsequent increased decomposition due to simulated grazing was rather weak or even negative (Fig. 7). We conclude that ^{15}N -transfer during the last month of our experiment was mainly via exudation

of N-rich compounds or rhizodeposition and not related to decomposition of belowground donor roots (and thus under control of the labelled donor individual).

The heterogeneous pattern of ^{15}N -transfer between monocultures and mixtures indicate a kind of plant behaviour *sensu* Karban (2008). Especially ^{15}N -transfer patterns of *Achillea* suggested self/non-self discrimination (Falik et al. 2003; Karban and Shiojiri 2009) or even kin recognition (Biedrzycki et al. 2010), with reduced belowground competition *within* the same species and enhanced resource competition *between* different species. In monocultures a kind of altruistic behaviour was observed: resources of a cut (and thus less fit) donor individual were distributed to neighbouring receiver individuals of the same species; maybe via enhanced root decomposition (although no connection was found to the degradation of decomposition standards). A contrary effect was observed in mixtures: ^{15}N -transfer from cut donors to neighbouring receivers was reduced for all species in 2- and 3-species-mixtures, although effects were only sometimes significant. We suggest that in cut donor individuals, the available N-resources within roots were used to rebuild itself instead of strengthening neighbouring individuals. This is in accordance to the finding of stimulated biomass production after defoliation via grazing or clipping (Sanford et al. 1995; Ayres et al. 2007), which seems to enhance internal N-remobilization (Thornton and Millard 1993) and overall nitrogen use efficiency (NUE). We identified highly interesting patterns, especially the interacting effects of species composition and disturbance on N-dynamics between functionally different individuals, which still need more investigation e.g. in relation to the mediating effect of grazing animals, which affect N-cycling not only by grazing but also by trampling and dropping of excrements (Vinther 1998; Moller Hansen et al. 2002). Additionally, simulated and real herbivory can alter plant responses differently as has been shown for metabolic processes and root growth dynamics (Hummel et al. 2007; Henkes et al. 2008).

Microcosms and communities

We found higher community productivity and nitrogen accumulation in mixtures with *Trifolium* which was driven by the legume itself (Fig. 6) and thus was due to the sampling effect (Huston 1997). *Trifolium* produced most biomass per individual in mixtures when it was released from intra-specific competition for above- or belowground space (which is a resource of itself, see Schenk et al. 1999) in mixtures (Fig. 3). Vigorous growth of the legume

species mainly proved an effective use of the small available soil volume and not as such an ecological response. Johnston & Pickering (2007) found a similar pattern for *Achillea millefolium* in a greenhouse experiment but no such effect on a *Poa* species. We neither observed high intra-specific competition for space in the forb nor in the grass species used in our study. Most communities were infected with vesicular-arbuscular mycorrhizal fungi (VAM), which indicated a high degree of belowground connectivity. Mycorrhizal fungi can have an important effect on overall plant-plant interactions (e.g. Hamel and Smith 1991; Moyer-Henry et al. 2006; van der Heijden and Horton 2009) and are capable of decomposition and transfer of organic compounds (Hodge et al. 2001). We cannot distinguish between transfer of ^{15}N -labelled nitrogen compounds via excretion and transport through the soil solution and a transport via hyphae of VAM. The trend to higher degradation of decomposition standards in communities with *Trifolium pratense* (Fig. 7) indicated higher belowground activity, which also has been found by Kreyling et al. (2008) for communities with legume species within a field experiment. Belowground activity can have significant effects on biomass and nitrogen accumulation (van der Heijden et al. 2008) and can affect plant performance via multiple pathways (De Deyn et al. 2003; Sanon et al. 2009).

CONCLUSION

Within this short-term microcosm experiment we were able to confirm positive effects of increasing species richness and legume presence on donor-receiver interactions as has been found in long-term field experiments (Spehn et al. 2002; Hector et al. 2007; Temperton et al. 2007; Marquard et al. 2009). We measured an increase in ^{15}N -transfer from monocultures to mixtures which was probably related to higher niche complementarity in mixtures. Additionally, we were able to elucidate some of the mechanisms of the role of species identity vs. species richness on donor-receiver interactions. The outcome depended strongly on the competitive ability and the resource use efficiency of receiver species. Both non-legume receivers (*Achillea millefolium*, *Phleum pratense*) profited from a legume donor in terms of biomass and nitrogen accumulation but if they grew in direct competition, the grass took significantly more advantage from N released by *Trifolium pratense* donors than the forb; confirming the better N-acquisition of grasses, which has been reported from field studies. We analyzed the effect of simulated grazing within three species richness levels and found that, while grazing had (as a trend) a positive effect on *intra-specific* ^{15}N -transfer from cut donors to shoots of receivers of the same species, it had (as a trend) a negative effect on *inter-specific*

transfer between cut donors and neighbouring receivers belonging to different species in mixtures. This is in line with the emerging knowledge about self/non-self and kin recognition in plants (Karban and Shiojiri 2009; Biedrzycki et al. 2010). The finding indicates a kind of intra-specific altruistic behaviour in response to grazing in monocultures whereas in mixtures, the available N-resources were remobilized internally to sustain the competitive strength of each species against the neighbouring other species. We now need more detailed investigations under (semi)-natural conditions, particularly related to the question how management regimes affect plant-plant interaction in an established sward.

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SUPPLEMENTARY MATERIAL

SPECIES INTERACTIONS ALONG AN N-AVAILABILITY GRADIENT IN A 3-MONTH GREENHOUSE STUDY

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HYPOTHESES

1. The positive effect of legume presence on neighbouring species (N-facilitation for receivers) will increase with decreasing N-availability in the substrate as predicted by the stress gradient hypothesis.
2. N-facilitation of legume species will change to competition because of reduced biological N₂-fixation (BNF) under high N-availability conditions.
3. Species from the functional group of grasses will profit more from N-facilitation than species from the functional group of forbs as is has been observed in (semi-)natural grassland habitats.

CONCLUSIONS

1. We found a slight increase in facilitation with decreasing N-availability in the substrate as predicted by the stress gradient hypothesis. The effect was most pronounced for the N-concentration in leaves but not detectable via the $\delta^{15}\text{N}$ natural abundance value, indicating, that N-facilitation occurred mainly via N-sparing in this short-term greenhouse study.
2. Strength and direction of the legume effect depended on the response parameter under investigation but it seemed (concluded from the interpretation of differences in legume parameters between low, medium and high N-availability) that legume species indeed used more soil N-resources under medium and high N-supply which implied higher competition especially in more diverse communities. Especially under medium N this seemed to have a negative effect on the N-concentration in forbs and grasses and led to decreasing $\delta^{15}\text{N}$ values. Lower $\delta^{15}\text{N}$ values indicate a more closed N-cycle.

3. Grasses profited more than forbs from a legume neighbour and increasing species richness, especially when soil N-resources were limited, reflecting superior N-capturing and N use efficiency as it has been observed in field experiments.

RESULTS AND DISCUSSION

ALL N-LEVELS TOGETHER

- Most variation in productivity, N-concentration [%], $\delta^{15}\text{N}$ and C:N ratios was explained by the number of individuals per community (Fig. 1, covariable: $F_{1,169} = 161.308$, $p < 0.001$) and by N-availability in the substrate (Table 1, $F_{2,169} = 11.56$ (NP_{ind}), 31-35 (N%, $\delta^{15}\text{N}$, C:N), $p < 0.001$). Effect of N-availability remained always highly significant irrespective of its fitting order in the model. Communities under medium N were most productive (Fig. 2). Biomass production under high N-availability might be limited because of low pH values due to fertiliser application (Table 1). N-concentrations and $\delta^{15}\text{N}$ values in plant leaves increased with increasing N-supply (Fig. 3).

→ Species performance within habitats which differ significantly in the N-availability in the substrate is predominantly under environmental control and not determined by positive or negative species interactions (Cardinale et al. 2009; Ma et al. 2010; Michalet et al. 2006). The same effect was found within this microcosms study, confirming that this general mechanism holds true at very different spatial scales.

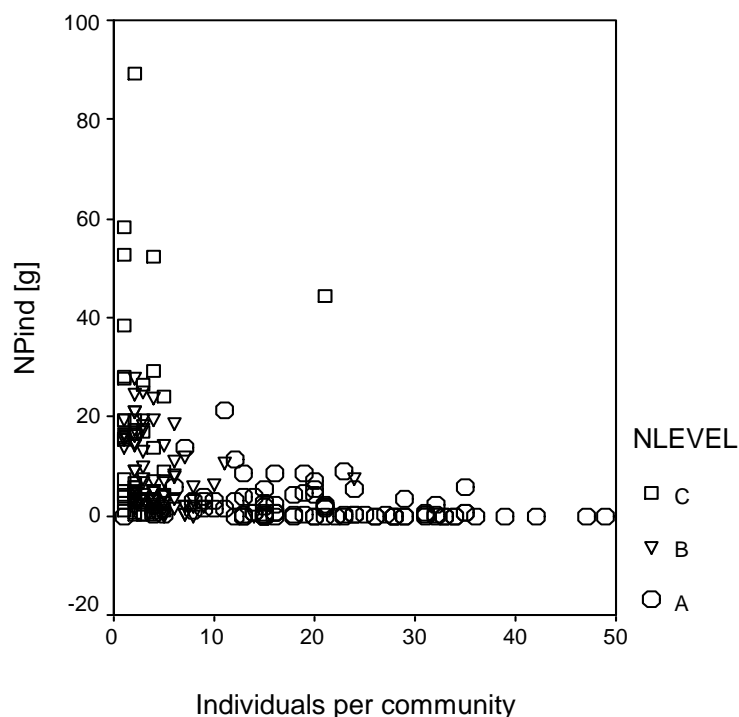


Figure 1 Effect of number per individuals on individual biomass (NP_{ind}) in N-level low (A), medium (B) or high (C). Most individuals grew in communities with low N-availability in the soil, whereas only few individuals grew (< 10) with high N-availability.

Table 1 Soil properties from the analyses of subsamples for substrates in all three N-levels: A = low N, B = medium N, C = high N. Indicated are mean values (± 1 standard deviation of the mean) for the N-concentration of the total soil N-pool (mineral and organic N-forms together), the $\delta^{15}N$ values for the same N-pool, pH values and the C:N ratios (both total soil C- and N-pools, mineral and organic forms together).

	n	N%	SD	$\delta^{15}N$	SD	pH	SD	C:N	SD
soil A_start	2	0,052	0,03	-4,8	0,73	6,8	0,06	31,6	6,46
soil A_end	9	0,054	0,02	-2,8	0,64	6,4	0,59	31,0	6,38
soil B/C_start without fert.	2	0,189	0,08	-1,4	0,58	6,5	0,02	33,1	2,76
soil B_bare at end	2	0,229	0,07	1,1	3,05	4,6	0,05	17,9	12,85
soil B_end	5	0,092	0,05	-2,3	0,97	5,0	0,46	26,0	8,13
soil B/C_start without fert.	2	0,189	0,08	-1,4	0,58	6,5	0,02	33,1	2,76
soil C_bare at end	2	0,493	0,48	2,0	2,76	4,5	0,33	9,5	8,84
soil C_end	3	0,417	0,22	2,1	1,13	4,6	0,08	9,3	3,10

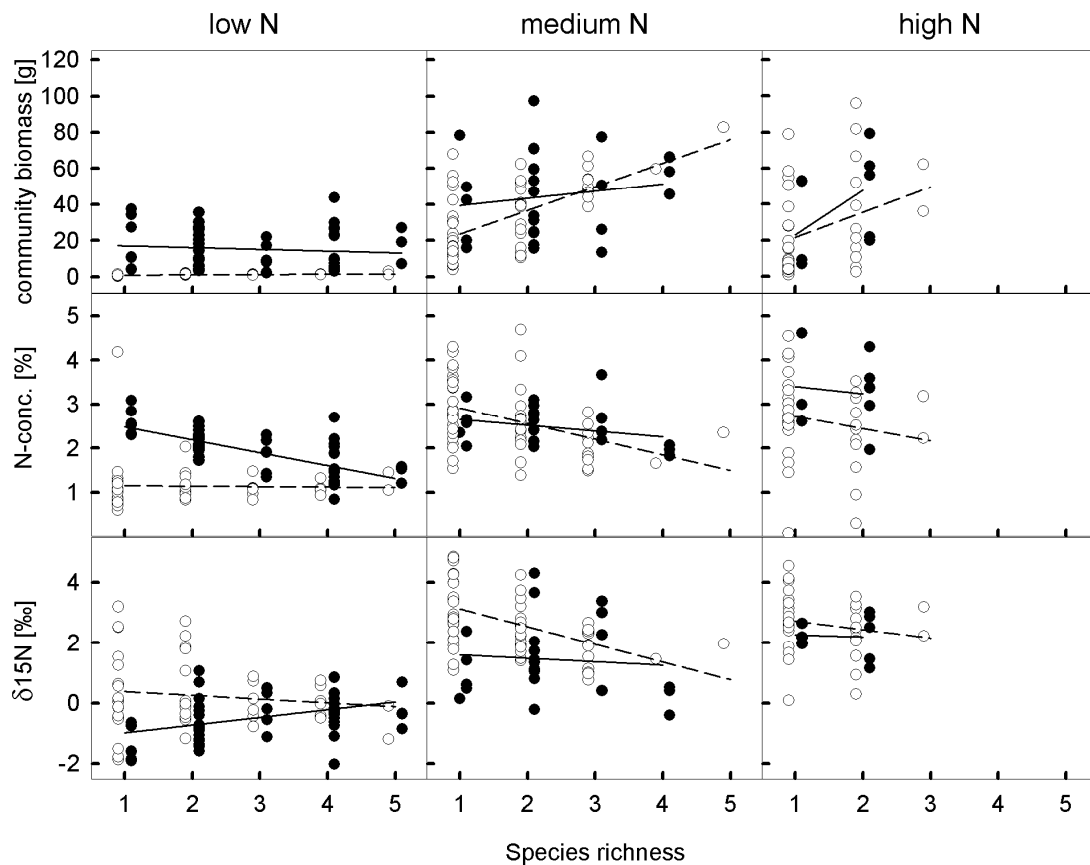


Figure 2 Effect of legume presence for community biomass (per pot) and mean values over all individuals per community for N-concentration and $\delta^{15}\text{N}$ values in plant leaves. Open symbols indicate communities without legume presence with a dashed linear regression line along the three N-levels, filled circles indicate communities with legume presence with a solid linear regression line along the three N-level.

- Effects of the other factors besides the N-availability in the substrate (species richness, functional group richness, functional identity and legume presence in communities) were mostly only significant ($p \leq 0.05$) if they were fitted first after the covariable in the model (2nd order). Effects of legume presence ($F_{1,169} = 16-84$, $p < 0.001$) or functional identity ($F_{2,169} = 6-58$, $p < 0.001$) had more explanatory power than functional group richness ($F_{2,169} = 3-15$, $p < 0.014$) or species richness ($F_{4,169} = 4-5$, $p < 0.005$) on response parameters if fitted at 2nd order. The effect of functional identity vanished for N%, $\delta^{15}\text{N}$ and C:N, but not for NP_{ind} , if it was fitted after legume presence. The same held true for the effect of species richness if it was fitted after functional group richness. Thus, further analyses were conducted separately for the three N-levels and functional group were displayed as separate entities.

- Within the three functional groups (forbs, grasses and legumes) number of individuals per community (Fig. 1) and N-supply (Fig. 3) had the strongest effects on all response parameters confirming results from analyses across all data together. All functional group-specific response parameters were mainly under environmental control but additional legume effects occurred.

→ Biodiversity experiments in the greenhouse are not very common and only few studies provide evidence of positive biodiversity effects *per se* on community biomass production (e.g. Lanta and Leps 2006). More often a predominant effect of legume species presence has been found (Mikola et al. 2002; Spaekova and Leps 2001). The importance of the sampling effect should increase with decreasing spatial scale (from field to greenhouse, Balvanera et al. 2006), thus, it was not surprising that the presence of legume species had stronger effect on response parameters than functional identity, functional group or species richness if tested at 2nd order over all N-levels.

- Legume presence had in general positive effects on N-concentration and C:N ratios for forbs and grasses (Fig. 3). Significant differences due to legume presence occurred in the low N-level for N-concentrations in forbs ($p = 0.022$) and in grasses ($p = 0.004$) and for the C:N ratio in grasses ($p = 0.001$) (Fig. 3).
- The increase in N-concentrations in leaf tissue (measured as per cent increase between forbs/grasses grew without and with legumes) is higher in the low N-level than in the medium N-level (low, medium (and high) N-levels: $\sim 33 > 28 (> 6)$ and $27 > 23 (> 20)$ % increase in forbs and grasses, respectively, Fig. 3).
- Legume presence had no significant effect on $\delta^{15}\text{N}$ values (mean over all species richness levels) in both forbs and grasses (Fig. 3).

→ N-facilitation increased slightly with increasing N-stress for receiver species, especially in terms of N-concentration in leaf tissue: whereas a positive legume effect was detectable under low N-availability, it vanished under medium or high N-availability.

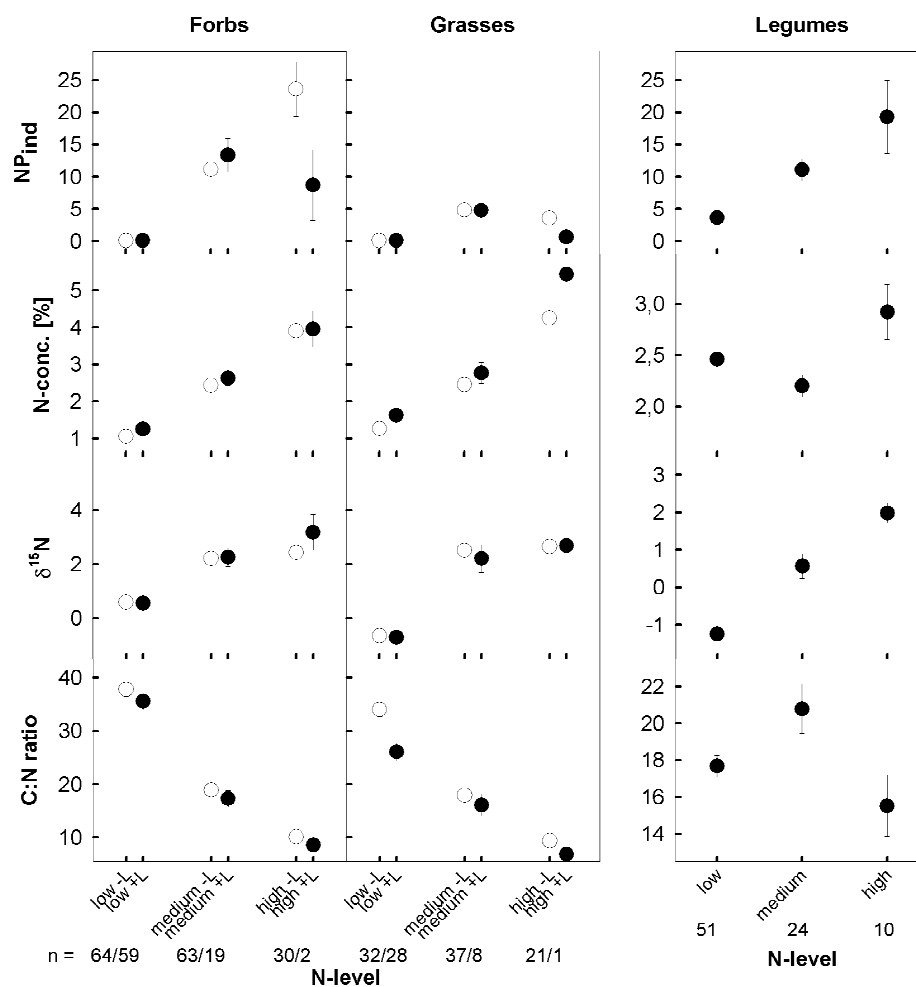


Figure 3 Effect of legume presence **along the N-gradient** on response parameters (mean values over all species richness levels within an N-level for individual biomass: NP_{ind} , N-concentration, $\delta^{15}N$ values and C:N ratios in leaf tissue) of the three functional groups forbs, grasses and legumes along. Open symbols represent mean over all individuals without a legume neighbour, closed symbols represent mean over all individuals with a legume neighbour, given are mean values of all species in all species richness levels per functional group ± 1 standard error of the mean, n = number of individuals within each group without/with legume as neighbour in communities).

N-LEVELS SEPARATED

- Along the gradient of species richness in three different N-levels (low = Fig. 4, medium = Fig. 5 and high = Fig. 6), response parameters changed differently with increasing N-supply in the substrate.
- Under low N-availability (Fig. 4), species richness had a positive effect on N-concentration (increased) and C:N ratios (decreased) in grasses whereas the effect was negative under medium N-supply (Fig. 5) and indifferent under high N-supply (Fig. 6).

→ Under low N-availability, higher species richness *per se* increased facilitation; maybe due to an increase in rhizodeposition and enhanced soil microorganism community with strongest

effects on the best competitors (grasses). When soil N-resources increased (medium N-level), the effect of increasing species richness changed from facilitation to competition: an increase in diversity led to decreasing N-concentrations and $\delta^{15}\text{N}$ values and increasing C:N ratios in forbs and grasses. The combination of these results indicates higher competition for N. In the four species mixtures, the legume species seemed to increase BNF due to higher resource competition: the increase in N-concentration with a decrease in C:N ratio was accompanied by a decrease in $\delta^{15}\text{N}$ values. Under high N-availability, competition for N-resources seemed to play only a minor role although even the legume species used soil N as indicated by high $\delta^{15}\text{N}$ values.

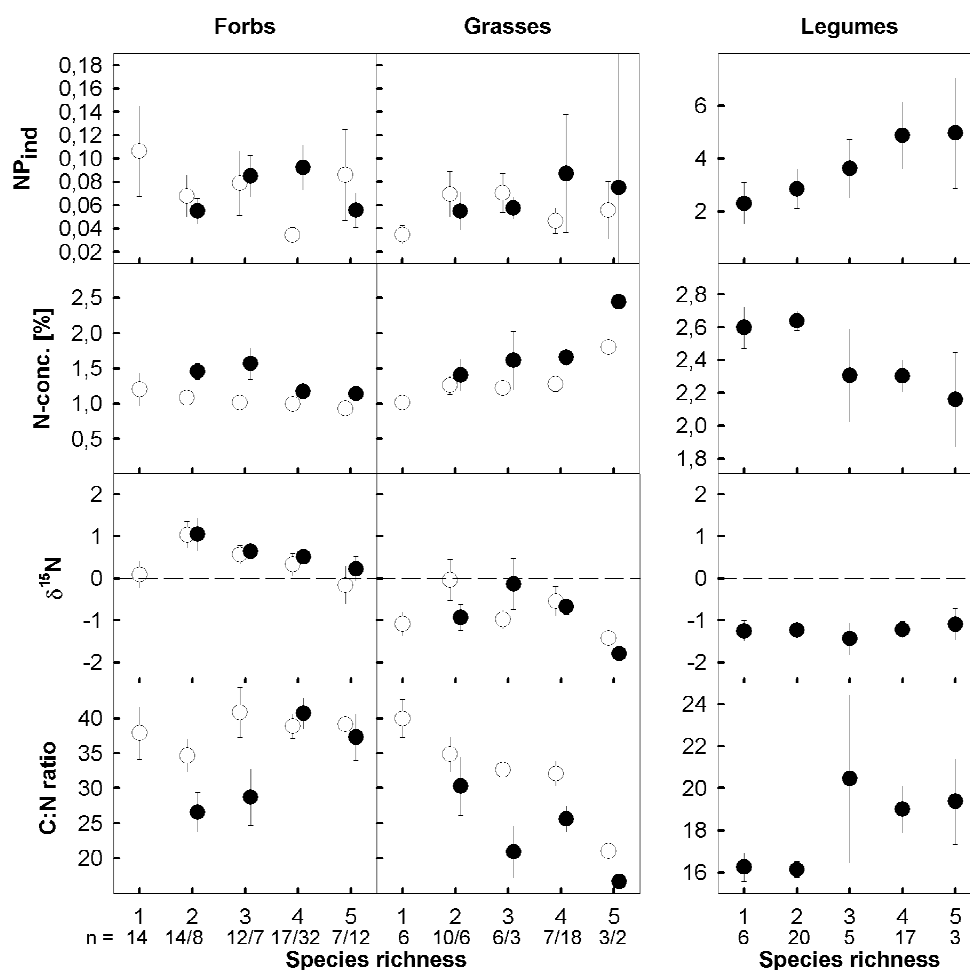


Figure 4 Effect of legume presence in the **low N-treatment** on response parameters (individual biomass: NP_{ind} , N-concentration, $\delta^{15}\text{N}$ values and C:N ratios in leaf tissue) of individuals of the three functional groups forbs, grasses and legumes along the species richness gradient. Open symbols represent individuals without a legume neighbour, closed symbols represent individuals with a legume neighbour, given are mean values of all species per functional group ± 1 standard error of the mean, n = number of individuals within each group without/with legume as neighbour in communities).

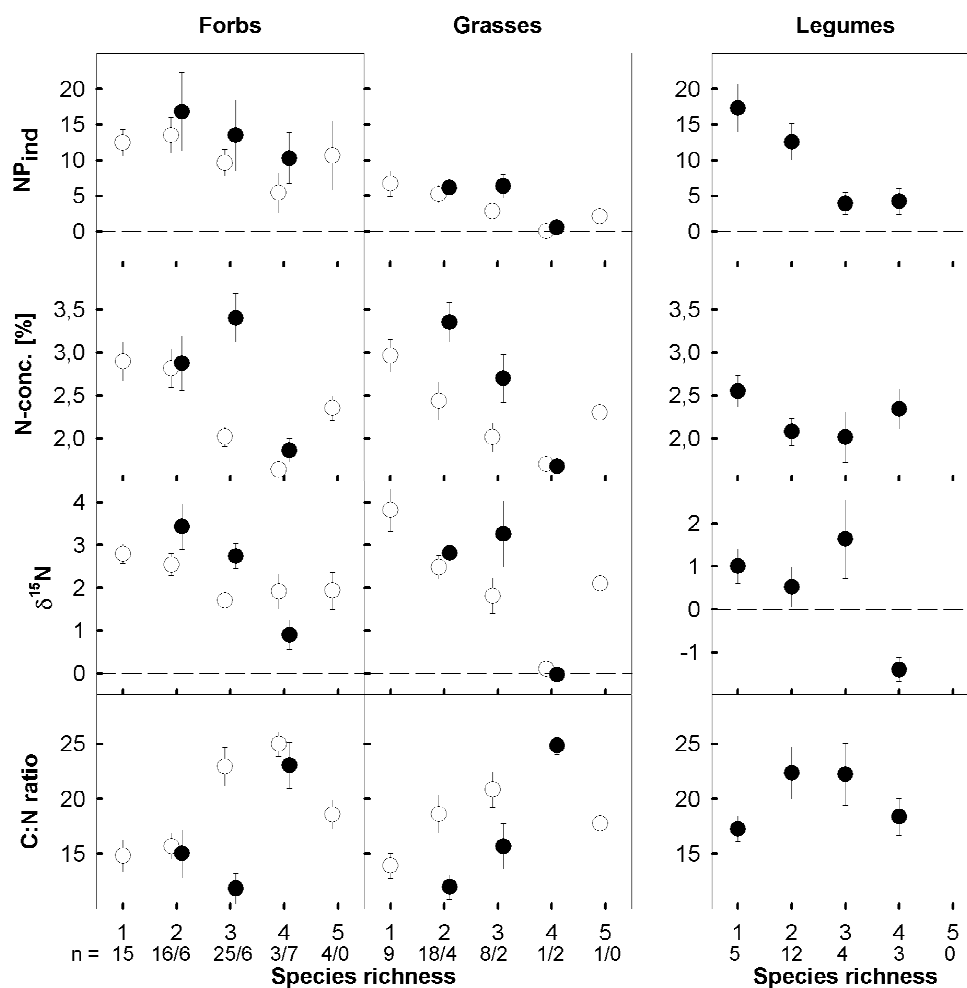


Figure 5 Effect of legume presence in the **medium N-treatment** on response parameters (individual biomass: NP_{ind} , N-concentration, $\delta^{15}N$ values and C:N ratios in leaf tissue) of individuals of the three functional groups (forbs, grasses and legumes) along the species richness gradient. Open symbols represent individuals without a legume neighbour, closed symbols represent individuals with a legume neighbour, given are mean values of all species per functional group ± 1 standard error of the mean, n = number of individuals within each group without/with legume as neighbour in communities).

- Legume presence enhanced N-concentration and lowered C:N ratios in leaves of forbs and grasses in all three N-levels (Figs 4-6) but effects were stronger in grasses than in forbs. It had no consistent effect on $\delta^{15}N$ values.

→ The increase in N-concentration accompanied with decreasing C:N ratios (especially in grasses) indicated a higher nitrogen use efficiency (NUE) when legumes were present in the communities.

→ Better NUE without similar homogeneous changes in $\delta^{15}N$ values showed, that the positive legume effect was **mainly attributed to N-sparing and not to N-transfer from legumes** (as N-donors) to neighbouring species (as N-receivers). N-sparing means, that soil N-resources

could be used more complete by non-fixing species if an N-fixing species *does not use it* because it sustained itself via biological nitrogen fixation (McNeill and Wood 1990a). Short-term N-transfer occurs as a field study with a ^{15}N -enriched tracer showed (Gylfadottir et al. 2007) but the effect might not be strong enough to change $\delta^{15}\text{N}$ values in receivers. A reason might be that the $\delta^{15}\text{N}$ value is *per se* an integrator of the N-cycle (Robinson 2001) and only severe treatments like the application of cattle urine affect the $\delta^{15}\text{N}$ natural abundance in the short term (Eriksen and Høgh-Jensen 1998).

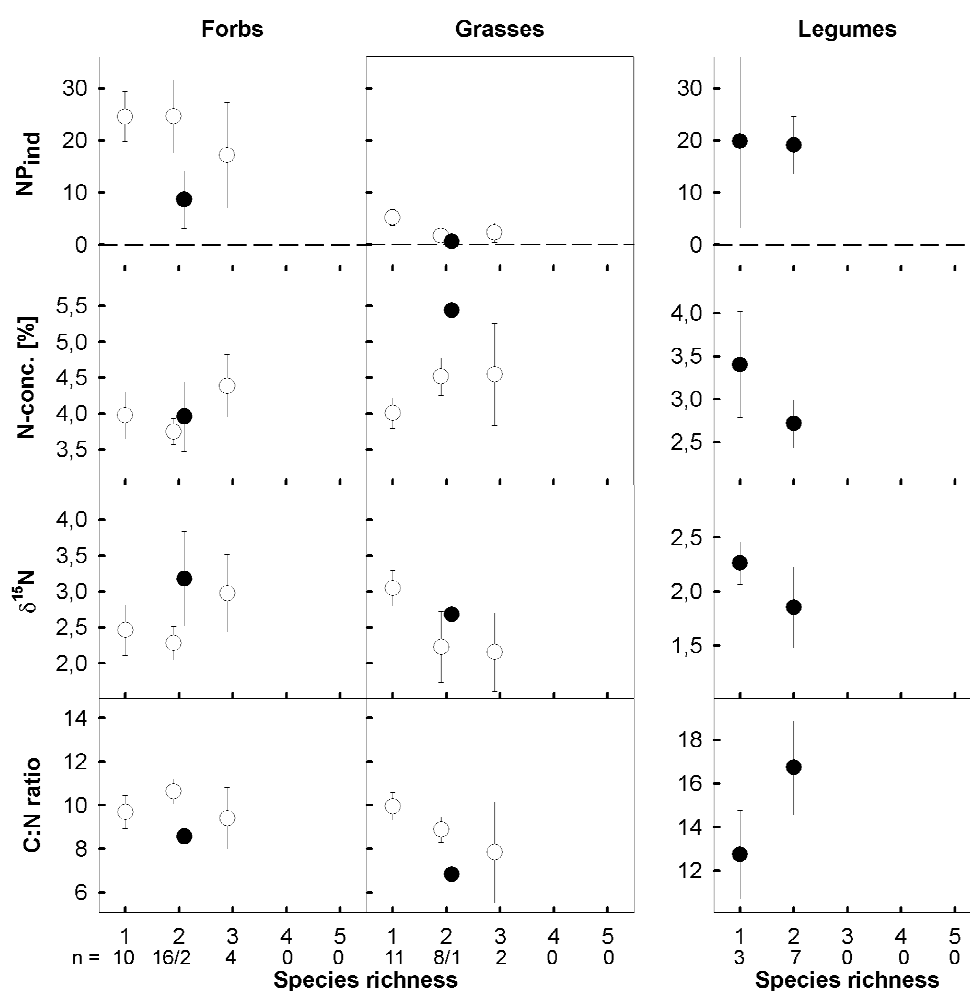


Figure 6 Effect of legume presence in the **high N-treatment** on response parameters (individual biomass: NP_{ind} , N-concentration, $\delta^{15}\text{N}$ values and C:N ratios in leaf tissue) of individuals of the three functional groups forbs, grasses and legumes along the species richness gradient. Open symbols represent individuals without a legume neighbour, closed symbols represent individuals with a legume neighbour, given are mean values of all species per functional group ± 1 standard error of the mean, n = number of individuals within each group without/with legume legume as neighbour in communities).

- Grasses respond stronger than forbs to the presence of legume species within communities. Especially N-concentrations and C:N ratios were consistently positive affected by a legume neighbour (Figs 4, 5).
- The changes in N-concentrations and C:N ratios were not mirrored in homogeneous changes in $\delta^{15}\text{N}$ values.

→ C:N ratios in grasses compared to forbs showed that grasses had a higher competitive strength for capturing available soil-N resources and exploit the soil volume quicker than forbs species especially in more diverse communities as it could be observed in field experiments. It is a kind of founder effect; a typical confounding factor for greenhouse experiments where the researcher determine time of species arrival and not e.g. phenology of different species.

MATERIALS AND METHODS

Biotic environment:

- Monocultures and mixtures, grown from seeds in the microcosms, of species from the functional group of N-fixing legumes, grasses or forbs (without separation in small and tall forbs) in three N-availability levels in the soil
- Communities grew ~90 days in the greenhouse during summer 2007
- Species were: 2 legumes, 3 grasses and 8 forbs
 - *Trifolium pratense* L.
 - *Lotus corniculatus* agg.
 - *Anthoxanthum odoratum* agg.
 - *Festuca pratensis* Huds. s. l.
 - *Phleum pratense* agg.
 - *Geranium pratense* L.
 - *Achillea millefolium* L.
 - *Matricaria inodora* L. , nom. illeg. (nom. superfl.) = *Tripleurospermum perforatum* (Mérat) Lainz
 - *Chrysanthemum leucanthemum* L. = *Leucanthemum vulgare* Lam. s. str.
 - *Prunella vulgaris* L.
 - *Hieracium pilosella* L.
 - *Leontodon autumnalis* L.
 - *Plantago lanceolata* L.

Abiotic environment:

- N-level were low, medium or high (~0.007, ~0.066 and ~0.206 % N_{total} in the substrate), realized by the 1:1 (v/v) mixture of 0-Erde-Sand (low N), ED73-Sand with low or high addition of slow-release/long-term fertiliser (NH₄NO₃) in medium and high N-levels
- We used 4 l pots, each N-level should contain 94 pots
- Pots were randomized on tables once per week to prevent edge effects
- Irrigation with tap water was performed automatically and manually if necessary
- We determine individuals, cover, height of highest individual and biomass per species during the experiment and at the time point of harvest
- Plant material were oven dried (60°C/> 60h), ground in stainless steel devices in a Retsch ball mill, packed in tin capsules and analyzed with an EA-IRMS (element analyzer-isotope ratio mass spectrometer) to determine N-concentration [%] and $\delta^{15}\text{N}$ natural abundance [‰] per sample and with an EA to determine C-concentration per sample

Statistics:

- We used ANCOVA (type I sum of squares) to determine effects of different factors, order of factors within the model were changed to find the most important factor for each response parameter, the covariable was always tested first
 - Covariable: number of individuals per community (= per pot), square root transformed
 - Independent variables (factor): N-level (N), species richness (SR), functional group richness (FG), legume presence in community (1/0; L), functional identity of species (FG-ID)
 - Dependent variables (response parameter): individual biomass (NP_{ind}) [g], N-concentration [%], $\delta^{15}N$ value [‰], C:N ratio
 - NP_{ind} and N [%] were square root transformed, C:N ratio log10-transformed and $\delta^{15}N$ values were not transformed to met the assumptions of normality (Kolmogorov-Smirnov Test) and of homogeneity of variances (Levene's Test)
- We used ANCOVA (type III sum of squares) to test for significant effects of N-level and legume presence within the functional groups (compare to Fig. 3)

Confounding effects:

- germination rate of seeds were rather low → use of individuals/pot as covariate in all ANOVAs
- additional planting of seedlings during the 2nd week of experiment was not successful
- problems with the automatic irrigation caused drought stress and high seedling mortality especially in the high N-treatment (→ no higher diversity level)
- N-levels had different pH values due to fertiliser application: low: ~5.5, medium: 4.5-5.0, high: 4.0-4.5 pH in substrate

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ERKLÄRUNG

Hiermit erkläre ich,

dass ich die vorliegende Dissertationsschrift selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

Hiermit erkläre ich,

dass ich weder die vorliegende noch eine gleichartige Doktorprüfung an einer anderen Hochschule endgültig nicht bestanden habe.

Jülich, den 12.04.2010

(gez. Lea Lucia Anna Martin)